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Effects of chlorophyllide *a* oxygenase overexpression on light acclimation in *Arabidopsis thaliana*

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Abstract

Land plants change the compositions of light-harvesting complexes (LHC) and chlorophyll (Chl) *a*/*b* ratios in response to the variable light environments which they encounter. In this study, we attempted to determine the mechanism which regulates Chl *a*/*b* ratios and whether the changes in Chl *a*/*b* ratios are essential in regulation of LHC accumulation during light acclimation. We hypothesized that changes in the mRNA levels for chlorophyll *a* oxygenase (CAO) involved in Chl *b* biosynthesis are an essential part of light response of Chl *a*/*b* ratios and LHC accumulation. We also examined the light-intensity dependent response of *CAO*-overexpression and wild-type *Arabidopsis thaliana* plants. When wild-type plants were acclimated from low-light (LL) to high-light (HL) conditions, *CAO* mRNA levels decreased and the Chl *a*/*b* ratio increased. In transgenic plants overexpressing *CAO*, the Chl *a*/*b* ratio remained low under HL conditions; thereby suggesting that changes in the *CAO* mRNA levels are necessary for those in Chl *a*/*b* ratios upon light acclimation. Under HL conditions, the accumulation of Lhcb1, Lhcb3 and Lhcb6 was enhanced in plants overexpressing *CAO*. On the contrary, in a *CAO*-deficient mutant, *chlorina 1-1*, the accumulation of Lhcb1, Lhcb2, Lhcb3, Lhcb6 and Lhca4 was reduced. In comparison to wild-type, β-carotene levels were reduced in *CAO*-overexpressing plants, while they were elevated in *chlorina 1-1* mutants. These results imply that the transcriptional control of *CAO* is a part of the regulatory mechanism for the accumulation of a distinct set of LHC proteins upon light acclimation.

Abbreviations: CAO – chlorophyllide *a* oxygenase; *ch* – *chlorina*; Chl – chlorophyll; CP – chlorophyll protein; LHC – light-harvesting protein complex; Lhca – light-harvesting chlorophyll *a*/*b*-protein of PSI; Lhcb – light-harvesting chlorophyll *a*/*b*-protein of PSII

Introduction

Photosynthetic organisms experience large fluctuations in the intensity of incident light which they receive in their dynamic natural environments. Acclimation to different light intensities involve a number of biochemical and morphological modifications; primarily involving the composition of the photosynthetic apparatus (Anderson et al. 1988; Melis 1991; Niyogi 1999). Adjustment of photosynthetic antenna size is one of the major strategies that higher plants employ for acclimation to variable light intensities (Chow and Anderson 1987; Anderson et al. 1988; Melis 1996; Murchie and Horton 1997, 1998).

Antenna systems of higher plants are classified into two groups: core complexes and peripheral light-harvesting complexes (LHC). Core complexes

are comprised of Chl *a,* β-carotene and several polypeptides that are encoded by the plastid genome, such as CP43 and CP47 (for a review, see Green and Durnford 1996). In contrast, LHC are made of Chl *a*, Chl *b*, xanthophylls and polypeptides that are encoded by nuclear genes belonging to the *Lhc* super-gene family (for a review, see Jansson 1999). In *Arabidopsis thaliana*, a total of 10 different LHC proteins are integrated in the antenna systems. These 10 proteins are further classified into three groups according to their respective locations within photosystems (PSs). Lhcb1, Lhcb2 and Lhcb3 are localized in the outermost part of PS II and comprise LHCII, the major peripheral antenna complexes of PS II and their composition ratios vary from 10:3:1 to 20:3:1(Machold 1991). Variations in composition ratios are dependent upon plant growth conditions and preparative methods for thylakoid membrane isolation (Machold 1991). LHCII is the most abundant pigment–protein complex within photosystems and it binds 70% of total Chls in PSII (Peter and Thornber 1991). Lhcb4, Lhcb5 and Lhcb6 comprise the minor antenna complexes that locate between LHCII and the core complex. Lhca1, Lhca2, Lhca3 and Lhca4 comprise the peripheral antenna complexes of PSI (Ben-Shem et al. 2003). It is likely that each LHC protein has a specific role, as genes encoding these proteins have been conserved during the evolution of green plants (Tokutsu et al. 2004). With the exception of Lhcb1 and Lhcb2, which are involved in state transition (Andersson et al. 2003), specific roles for each LHC proteins are not known.

A few regulatory mechanisms for LHC accumulation have been proposed. Transcriptional control of *Lhc* gene expression is often regarded as a major regulatory mechanism of LHC accumulation. Actually, it is well established that the steady-state mRNA levels for LHC are dependent upon envionmental light intensities (e.g. Teramoto et al. 2002; Masuda et al. 2003). The repression of a *Lhc* gene by the antisense RNA technique lead to decreased accumulation of the corresponding LHC protein in antisense plants for *Lhca4* (Zhang et al. 1997), *Lhca2*, *Lhca3* (Ganeteg et al. 2001), *Lhcb1, Lhcb2* (Andersson et al. 2003; Ruban et al. 2003), *Lhcb4* and *Lhcb5* (Andersson et al. 2001). However, in spite of these observations which implied that LHC accumulation is controlled by transcriptional regulation, several lines of evidence indicate that other regulatory mechanisms may play an important role in LHC accumulation. For example, in tobacco *Lhcb1* antisense plants, 95% repression of *Lhcb1* mRNA levels did not affect accumulation of LHC proteins (Flachmann and Kühlbrandt 1995; Flachmann 1997). A similar observation was reported with *Arabidopsis* antisense plants for *Lhcb2* (Andersson et al. 2003). In addition to our knowledge, there is no previous report which has demonstrated that the overexpression of *Lhc* genes results in an increased accumulation of LHC proteins.

In addition to transcriptional regulation, it is interesting to consider the additional mechanisms that may function to regulate the abundance of LHC proteins in response to light intensities. It has been proposed that availability of Chl *b* (Bellemare et al. 1982; Harrison and Melis 1992) plays a role controlling LHC accumulation and consequently the antenna size. For example, a barley Chl-*b* less mutant, *chlorina-f2*, has only 50 Chl *a* molecules in its PSII antenna, whereas WT has about 250 Chl *a* and *b* molecules in its PSII antenna (Ghirardi et al. 1986). According to this model, LHC apoproteins that bind Chl *b* are stably inserted into thylakoid membranes and excess LHC apoproteins that fail to bind Chl *b* may be degraded by proteases (Lindahl et al. 1995; Yang et al. 1998, 2000) or sorted out by vesicle transportation (Hoober and Eggink 2001). Data from several independent studies support this model. (1) In addition to changes in LHC accumulation, changes in Chl *a*/*b* ratios in response to light intensities are observed in a wide range of land plants (e.g. Murchie and Horton 1997). (2) LHC accumulation was decreased in Chl-*b* less plants described as above. (3) A slight increase (10–20%) in LHCII accumulation was observed with transgenic *Arabidopsis* plants that showed lower Chl *a*/*b* ratios (Tanaka et al. 2001). (4) A zinc derivative of Chl *b* promotes insertion and stabilization of LHC precursor proteins into plastid membranes, while a zinc derivative of Chl *a* does not (Kuttkat et al. 1997).

The mechanism that determines Chl *a*/*b* ratios is also unclear. Since Chl *b* is synthesized from Chl *a* by the action of chlorophyllide *a* oxygenase (CAO), several authors speculated that expression of the *CAO* gene determines Chl *a*/*b* ratios. Masuda et al. (2002) reported that Chl *a*/*b* is correlated with the steady state mRNA levels for

CAO in a green alga, *Dunaliella salina*, under LL (50 μmol m^{-2} s^{-1}) and HL (2200 μmol m^{-2} s^{-1}). Harper et al. (Harper et al. 2004) also reported that the mRNA and proteins levels are correlated with Chl *a*/*b* ratios during the shift from moderate light (220 μmol m^{-2} s^{-1}) to shaded conditions (45 μmol m^{-2} s^{-1}) in *Arabidopsis thaliana*. However, it has not yet been determined whether only the changes in *CAO* expression cause the changes in Chl *a*/*b* ratios, or if other unidentified factors play more essential roles in the changes in Chl *a*/*b* ratios.

In this study, we examined the effects of CAO-overexpression in transgenic *Arabidopsis* plants. By using transgenic plants, we were able to distinguish the effects on Chl *a*/*b* ratios of altered *CAO* mRNA levels and those of other factors that may be altered under various light intensities. We demonstrated that the steady-state *CAO* mRNA levels have the predominant effects on Chl *a*/*b* ratios. Further, we also evaluated accumulation of 10 different LHC proteins in these plants in order to gain insight into the regulatory mechanism of LHC accumulation.

We should note that effects of CAO-overexpression on Chl *a*/*b* ratios and LHC accumulation were recently examined in tobacco *CAO* overexpressing plants (Pattanayak et al. 2005), independently from our group. They concluded that changes in Chl *a*/*b* ratios were not correlated with LHC accumulation, which is opposite to our conclusion. Discrepancy between their and our results was discussed as well.

Materials and methods

Plant material and growth conditions

For RNA and protein blotting analyses, *Arabidopsis thaliana* seedlings (Columbia ecotype) were grown under continuous illumination in a chamber equipped with natrium lamps (NH360F, Toshiba, Tokyo, Japan) at a light intensity of 100–1200 μE m^{-2} s^{-1} at 22 °C on a 1:1 mixture of vermiculite and nourished soil (Sankyo-Baido soil, Hokkai-Sankyo Co. Ltd., Kita-Hiroshima, Japan). For the overexpression of chlorophyllide *a* oxygenase (CAO), we fused the cauliflower mosaic virus 35S promoter with a full-length cDNA clone for *Arabidopsis* CAO in frame and introduced it into either the Columbia ecotype of *Arabidopsis thaliana*, or into a *CAO*-deficient mutant, *ch1-1*, as previously described in Tanaka et al. (2001). Two homozygous transgenic lines (YK1-3 and YK1-10) of WT background and another (YK2-3) of *ch1-1* background were selected for further analysis.

Northern blot analysis

Using a RNeasy Plant Mini kit (Qiagen GmbH., Hilden, Germany), total RNA was extracted from 3-week-old seedlings grown under continuous illumination from natrium lamps (NH360F) at 22 °C as described above. Utilizing standard methods, 2 μg total RNA was loaded per lane on denaturing RNA gels and transferred onto nylon membranes (Hybond N+, Amersham Bioscience Corp. NJ).

A PCR fragment spanning the entire open reading frame was amplified from a *CAO* cDNA clone (Tanaka et al., 2001) and an EST clone for Lhcb1 (98N12T7) were used to generate ^{32}P labeled DNA probes with a Takara Labeling Kit ver. 2 (Takara Shuzo Co., Shiga, Japan). Hybridization was carried out in Church hybridization buffer (0.5 M sodium phosphate buffer pH 7.2, 1 mM EDTA, 7% SDS) at 65 °C overnight. Membranes were washed with a high-stringency wash buffer (0.1 × SSC: [1 × SSC is 0.15 M NaCl and 0.0015 M sodium citrate], 0.1% SDS) at 65 °C for 10 min (3 times), exposed to phosphor-imaging plates (Fuji Film, Tokyo, Japan) and analyzed with a BAS1500 phosphor-image analyzer (Fuji Film).

Pigment analysis

Chlorophylls were extracted from leaves with acetone and their concentrations were measured according to the molecular coefficient published by Porra et al. (1989). Carotenoids were extracted from leaves with acetone and measured by HPLC according to the method of Masamoto et al. (1993) with slight modifications. The extracts were loaded on a C18 column (YMC AL303, 250 × 4.6 mm, 5 μm, YMC Co. Ltd., Kyoto, Japan) and eluted with an isocratic flow of 100% methanol for 17 min, followed by a mixture of 60% methanol, 20% ethanol and 20% hexane for 8 min at a flow rate of 1.2 ml min^{-1}. The eluates were monitored by a spectrophotometer, SPD-10AV (Shimadzu

Co. Ltd, Kyoto, Japan) at a wavelength of 450 nm. Pigments were identified and quantified by using standards that were purchased from DHI Water and Environment (Hoersholm, Denmark).

Protein immunoblots

Total protein was extracted from leaf material by grinding with solubilization buffer (56 mM Na_2CO_3, 56 mM dithiothreitol, 2% SDS, 12% sucrose and 2 mM EDTA) and with subsequent centrifugation at 10,000 *g* at 4 °C. Supernatants were collected and each sample was first diluted to 32 ng/μl Chl *a* with solubilization buffer. For proteins extracted from low-light grown WT plants, serial dilutions of 64, 32, 16 and 8 ng/μl Chl *a* were made and used as standards for quantification as described below. Ten μl of each diluted sample was loaded onto a 14% polyacrylamide gel, separated with electrophoresis and subsequently electro-transferred onto Hybond-P membranes (Amersham Biosciences Corp.). All the anti-LHC sera were obtained from AgriSera (Vannas, Sweden), except for anti-Lhcb4 antiserum which was a gift from Prof. K. K. Niyogi (University of California). Chemiluminescent detection was performed with the anti-LHC antisera using the ECL Plus Starter Kit (Amersham Biosciences Corp.) according to the manufacturer's instructions. Chemiluminesence of the immunologically detected signals was captured by a scanner and analyzed using the public domain ImageJ program (developed by Wayne Rasband at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij/). Signal intensities of each band were calculated according to the standard calibration curves prepared for each membrane using signals from serial dilutions with the WT extracts as describe above.

Results

Changes in Chl a/b *ratios and* CAO *mRNA levels upon shifts from HL to LL or LL to HL intensities*

We examined whether *CAO* mRNA levels change in response to light intensities in accordance to the changes in Chl *a*/*b* ratios in our experimental conditions. We transferred 3-week-old HL (1200 μmol m^{-2} s^{-1})-acclimated wild-type (WT) *Arabidopsis* plants (see Materials and methods) to LL (100 μmol m^{-2} s^{-1}) conditions and maintained them under LL for 3 d. During this period, the Chl *a*/*b* ratios decreased gradually from 4.02 to 3.70 (Figure 1a) and were thereby indicative of an increase in Chl *b* biosynthesis. The mRNA levels for *CAO* decreased within the first 8 h and then increased to 150% of the initial level (Figure 1b). We also examined the levels of *Lhcb1* mRNA in order to monitor the light-dependent control of *Lhc* gene expression. Changes in *Lhcb1* mRNA levels followed the same pattern as *CAO* mRNA; *Lhcb1* mRNA slightly decreased in the first 8 h after transfer to LL and then increased to 150% of the initial level (Figure 1c).

We also transferred LL-grown 3-week-old plants to HL conditions and kept them in the same conditions for 3 d. Chl *a*/*b* ratios increased from 3.41 to 4.22 and showed a decrease in Chl *b* accumulation. The mRNA levels for *CAO* drastically decreased to approximately 20% of the initial level in the first 8 h and slightly increased and again decreased to 20% of the initial level after 3 d of the transfer (Figure 1a). The mRNA levels for *Lhcb1* followed similar patterns as those of *CAO*. They dropped drastically in the first 8 h of HL exposure, slightly recovered and then finally reached 50% of the initial level (Figure 1b).

The steady-state *CAO* and *Lhcb1* mRNA levels were consistent with the Chl *a*/*b* ratios before and after acclimation to different light intensities. In our study, we used a wider light range from 100 to 1200 μmol m^{-2} s^{-1} and obtained similar results to those of Harper et al., (2004) in which they used low (95 μmol m^{-2} s^{-1}) to moderate (230 μmol m^{-2} s^{-1}) light intensities.

Correlation of the steady-state CAO *mRNA levels and Chl* a/b *ratios under various light conditions*

We previously demonstrated that *CAO*-overexpression resulted in a slightly decrease of Chl *a*/*b* ratios under low-light conditions in transgenic plants that were grown on agar plates (Tanaka et al. 2001). However, under different light conditions, the activities of various metabolic pathways, and/or expression patterns of *Lhc* genes may change. Alterations of these factors may give stronger effects on Chl *a*/*b* ratios than overexpression of *CAO*. Thus, we assessed whether the *CAO* mRNA levels primarily control Chl *a*/*b*

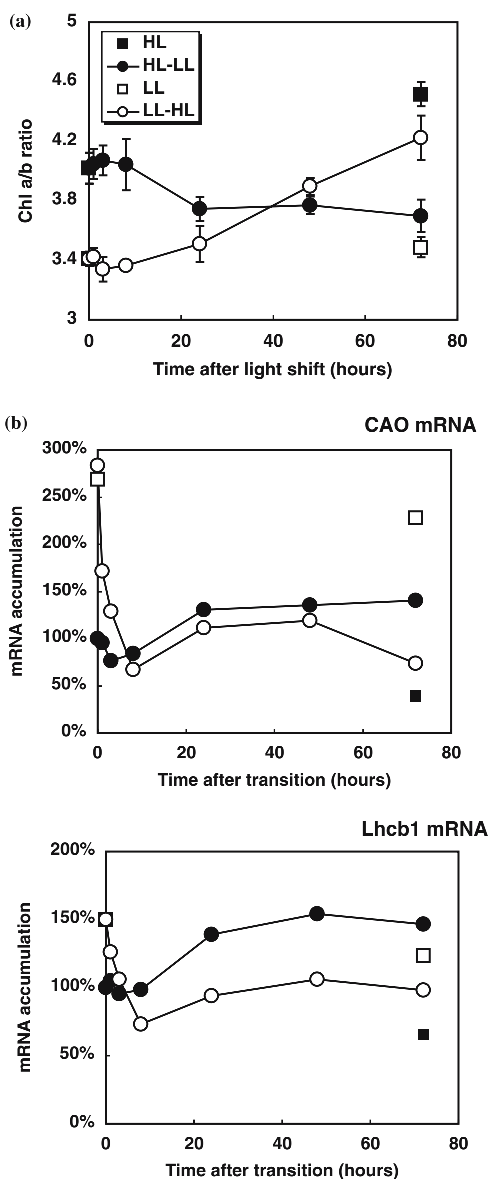


Figure 1. Coordinated changes in Chl *a*/*b* ratio and *CAO* mRNA accumulation upon light-shift. For the LL to HL shift experiments, three-week-old seedlings grown in LL conditions (100 μmol m^{-2} s^{-1}, open circles) conditions were transferred to HL conditions. For the HL to LL shift experiments, two-week-old seedlings grown in LL conditions were acclimated to HL (1200 μmol m^{-2} s^{-1}, solid circles) by increasing light intensities in a stepwise manner up to 1200 μmol m^{-2} s^{-1} in 1 week, and then transferred directly to LL conditions. Data from seedlings grown under continuous LL conditions (open squares) or those acclimated to HL conditions (solid squares) are also shown in graphs. $n = 4$. (a) Changes in Chl *a*/*b* ratio in WT *Arabidopsis* plants. Averages of four measurements are shown. (b) Northern blot analysis of *CAO* mRNA levels after light shifts. Total RNA indicated was analyzed by using a ^{32}P-labeled *CAO* cDNA probe. (c) Northern blot analysis of *Lhcb1* mRNA levels after light shifts. Total RNA at the time indicated was analyzed with hybridization of a ^{32}P-labeled *Lhcb1* cDNA probe.

ratios under various light intensities by using *CAO*-overexpression transgenic plants in which *CAO* transcription was driven by the cauliflower

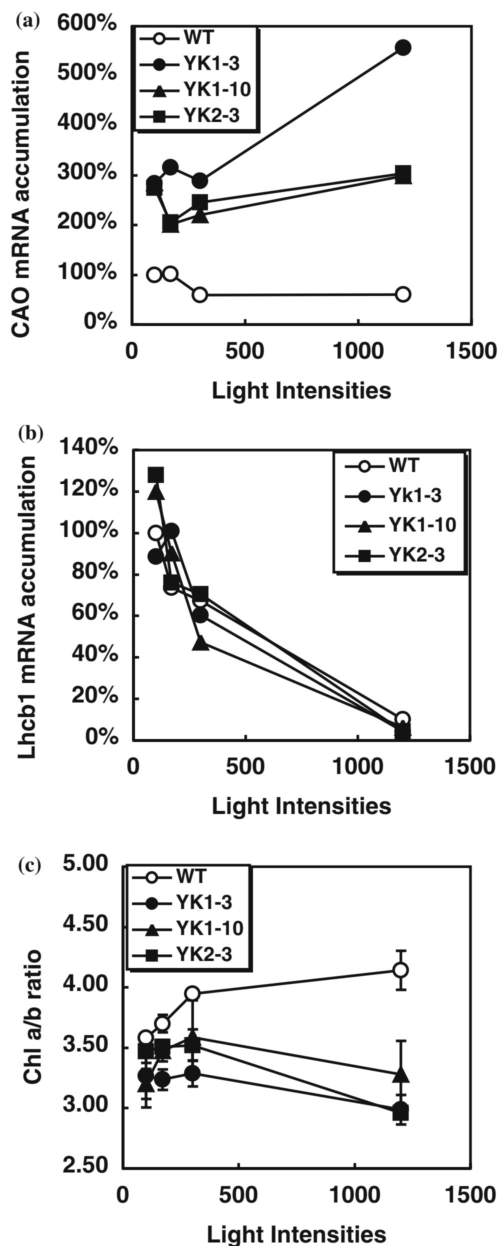


Figure 2. Effects of *CAO*-overexpression on Chl *a*/*b* ratios. Northern blot analysis of (a) *CAO* and (b) *Lhcb1* mRNA and in WT (open circles) and *CAO*-overexpression plants (YK1-3, solid circles; YK1-10, solid triangles; YK2-3, solid square) acclimated to various light intensities indicated on the graph. (c) Chl *a*/*b* ratios in WT and *CAO*-overexpression plants acclimated to various light intensities indicated on the graph. $n = 3$.

35S promoter previously described (Tanaka et al. 2001).

We grew three independent *CAO*-overexpression lines, YK1-3, YK1-10 and YK2-3, for 2 weeks under LL (100 μmol m^{-2} s^{-1}) and allowed them to acclimate to moderate-light (170 and 300 μmol m^{-2} s^{-1}) or HL intensities (1200 μ mol m^{-2} s^{-1}) for 1 week. In comparison to WT, *CAO* mRNA levels increased two- to ten-fold in the *CAO*-overexpression lines. In WT, *CAO* mRNA levels decreased by 50% under HL compared to those in 100 or 170 μmol m^{-2} s^{-1}. On the contrary, *CAO* mRNA levels did not decrease in YK 1-10 nor YK2-3 and increased in YK1-3 (Figure 2a). In contrast, *Lhcb1* mRNA levels in WT and the *CAO*-overexpression lines were not distinguishable between each other and they decreased in response to an increase of light intensities (Figure 2b). In the *CAO*-overexpression lines, Chl *a*/*b* ratios were highly correlated with the *CAO* mRNA levels (Figure 2c). They were increased up to 3.5 at moderate light intensities and decreased to 3.0–3.3 under HL in *CAO*-overexpression plants, while they were elevated to 4.2 under HL in WT plants. These results indicate that the expression of *CAO* has predominant control of the Chl *a*/*b* ratio; a control which may override the influence from other factors that may change in response to variation of light intensities.

Accumulation of LHC proteins in the CAO-*overexpression plants*

Under LL (100 μE m^{-2} s^{-1}) and HL (1200 μE m^{-2} s^{-1}) conditions, we examined the relationships of the Chl *a*/*b* ratios and the accumulation of LHC proteins by using plants which exhibit various levels of Chl *a*/*b* ratios; namely, WT, *CAO*-overexpression YK1-10 and CAO-deficient *ch1-1* plants. *ch1-1* is a mutant plant that has a 31-bp deletion in its *CAO* gene (Oster et al. 2000) and is rendered unable to synthesize Chl *b*.

Though most of the LHC proteins gave a single band in our immunoblotting analysis, both Lhcb4 and Lhca4 produced double bands. Lhcb4 protein showed a unique two-band migration pattern in the SDS-PAGE analysis (Figure 3) with the lower band being significantly intensified under HL. Similarly, two bands were observed under LL, however, the intensities of the lower bands were much fainter than those obtained from HL conditions. As Lhcb4 is encoded by a multiple gene family (Jansson 1999), it is possible that the multiple bands may have resulted from differential expression of a multiple gene family. For quantification purposes, we summed the intensities of these two bands and used them as a representation for Lhcb4. Similar to Lhcb4, immunoblotting analysis of Lhca4 also produced two bands. It is possible that the appearance of two bands is due to an artefact during the preparation of protein samples from leaves. The migration positions of these two bands were not identical to those of other Lhc proteins; the migrated position of the upper band was slightly higher than that of Lhca1 and the lower band was slightly lower than that of Lhca1 (data not shown). The relative band intensities between samples were also different from those of any other LHC proteins. Thus, it is not likely that one of the two bands was from the cross reaction of the Lhca4 antibody to another LHC protein, and we, therefore, considered both of the bands represented Lhca4.

Quantities of LHC proteins that were detected with protein blot analyses were compared for WT and YK1-10. Under LL, there were no significant differences detected, however, under HL, the levels of Lhcb1, Lhcb3 and Lhcb6 significantly increased in YK1-10 by 74, 40 and 83%, respectively. The levels of other LHC proteins were similar between WT and YK1-10.

In addition, the levels of LHC proteins between WT and *ch1-1* were also compared. In contrast to WT, the levels of Lhcb1 and Lhcb6 in *ch1-1* were significantly reduced by 77 and 71%, respectively under LL conditions. Under HL, the levels of

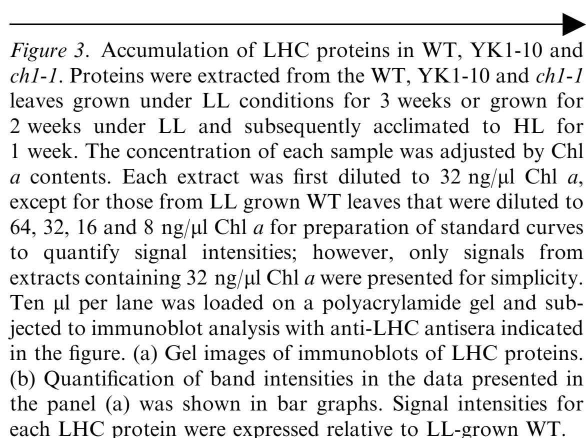
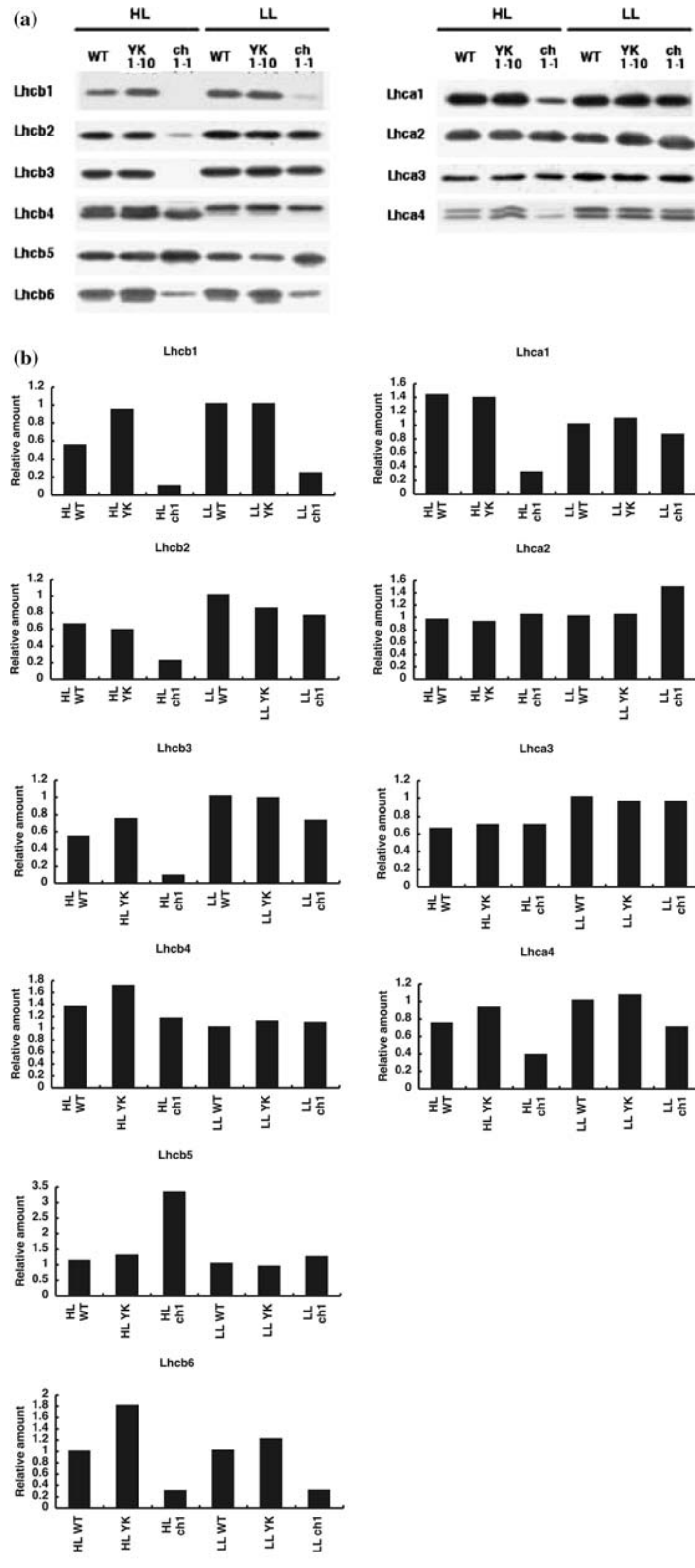


Figure 3. Accumulation of LHC proteins in WT, YK1-10 and *ch1-1*. Proteins were extracted from the WT, YK1-10 and *ch1-1* leaves grown under LL conditions for 3 weeks or grown for 2 weeks under LL and subsequently acclimated to HL for 1 week. The concentration of each sample was adjusted by Chl *a* contents. Each extract was first diluted to 32 ng/μl Chl *a*, except for those from LL grown WT leaves that were diluted to 64, 32, 16 and 8 ng/μl Chl *a* for preparation of standard curves to quantify signal intensities; however, only signals from extracts containing 32 ng/μl Chl *a* were presented for simplicity. Ten μl per lane was loaded on a polyacrylamide gel and subjected to immunoblot analysis with anti-LHC antisera indicated in the figure. (a) Gel images of immunoblots of LHC proteins. (b) Quantification of band intensities in the data presented in the panel (a) was shown in bar graphs. Signal intensities for each LHC protein were expressed relative to LL-grown WT.


(a)
HL
LL
WT
YK 1-10
ch 1-1
Lhcb1
Lhcb2
Lhcb3
Lhcb4
Lhcb5
Lhcb6
Lhca1
Lhca2
Lhca3
Lhca4
(b)
Relative amount
HL WT
HL YK
HL ch1
LL WT
LL YK
LL ch1

Lhcb1, Lhcb2, Lhcb3, Lhcb6, Lhca1 and Lhca4 were also significantly reduced in *ch1-1* by 83, 68, 85, 71, 79 and 49%, respectively. In contrast, the levels of Lhcb5 increased by nearly 3 times in *ch1-1* under HL in comparison to those in WT.

Carotenoid compositions in the CAO-*overexpression plants*

CAO-overexpression resulted in greater accumulation of a few LHC proteins under HL (Figure 3). These LHC proteins bind not only to Chls, but also to several carotenoids as well (Peter and Thornber 1991). It is possible that changes in LHC contents lead to altered carotenoid accumulation in *CAO*-overexpression plants. In order to examine the effects of *CAO*-overexpression in more detail, we compared carotenoid contents in WT, YK1-10 and *ch1-1* under HL. Because carotenoid contents are developmentally regulated (e.g., Tanaka et al. 1999), we examined them in leaves that were harvested from different developmental stages. We also examined the developmental changes in Chl *b* accumulation for an additional level of comparison to carotenoids (Figure 4a). We found that the Chl *b* levels did not change with leaf age. The levels of Chl *b* relative to total Chl contents were always higher in YK1-10, irrespective of the age of leaves.

The levels of neoxanthin (Figure 4b), lutein (Figure 4c), β-carotene (Figure 4d), xanthophylls cycle pigments (violaxanthin, antheraxanthin and zeaxanthin; Figure 4e) and the de-epoxydation rates were also all independent of leaf ages in WT and YK1-10. In contrast, the neoxanthin and lutein levels decreased and the de-epoxydation rates increased in an age-dependent manner in the *ch1-1* mutant.

There was no significant difference in the levels of neoxanthin, lutein and xanthophylls cycle pigments between WT and YK1-10. However, the levels of β-carotene and the de-epoxydation rates were significantly reduced in YK1-10 in comparison to WT. The greatest difference in the β-carotene levels between WT and YK1-10 was 25% in the third whorl, while the difference in the de-epoxydation rates was 0.11 in the fifth whorl. Neoxanthin levels in *ch1-1* was almost as twice as those of WT or YK1-10 in the first whorl of leaves, while in the seventh whorl, it was almost as half as those of WT or YK1-10. In comparison to WT, the levels of lutein, β-carotene, xanthophyll cycle pigments and the de-epoxydation rates were always higher in *ch1-1*. The difference in the de-epoxydation rates in the seventh whorl was remarkably different between WT or YK1-10 and *ch1-1*. While the rate was only 0.18 in WT and 0.16 in YK1-10, the calculated rate of 0.67 in *ch1-1* indicated that a substantial portion of the xanthophyll cycle pigments was de-epoxydated.

Discussion

Correlation between the CAO *mRNA levels and Chl* a/b *ratios*

The light intensity dependent changes in *CAO* mRNA levels observed in this study (Figures 1b and 2a) were also reported in a green alga, *Dunaliella salina*, upon shifts between HL and LL (Masuda et al. 2002, 2003). These observations suggest that the changes in *CAO* mRNA accumulation during light acclimation is a common response of green algae and land plants.

Shortly after the plants were transferred to different light conditions, we observed sharp drops in the *CAO* and *Lhcb* mRNA levels (see Figures 1b and c). Although the explanation for this observation is not clear, it is possible that the cells responded to the sudden change in their environment and increased the expression of "emergency" genes; such as those encoding heat shock proteins, which may have lead to a suppression of other gene expressions (Rossel et al. 2002; Kimura et al. 2003). It is also possible, even though we grew plants under continuous illumination, which conditions usually do not induce synchronized circadian oscillation, H-L shifts may have reset the intrinsic circadian clock, resulting in down-regulation of mRNA levels up to 8 h.

Harper et al. (2004) reported that *CAO* mRNA levels correlated to its protein levels in a low (45 μmol m^{-2} s^{-1}) to moderate (230 μmol m^{-2} s^{-1}) light range. Our observation of the correlated changes in Chl *a*/*b* ratios and *CAO* mRNA levels in higher light intensities up to 1200 μmol m^{-2} s^{-1} (see Figures 1 and 2) also indicated a similar correlation of CAO proteins and mRNA up to a light intensity of 1200 μmol m^{-2} s^{-1}.

The observed light-intensity dependent changes in mRNA levels were not unique to *CAO*. The

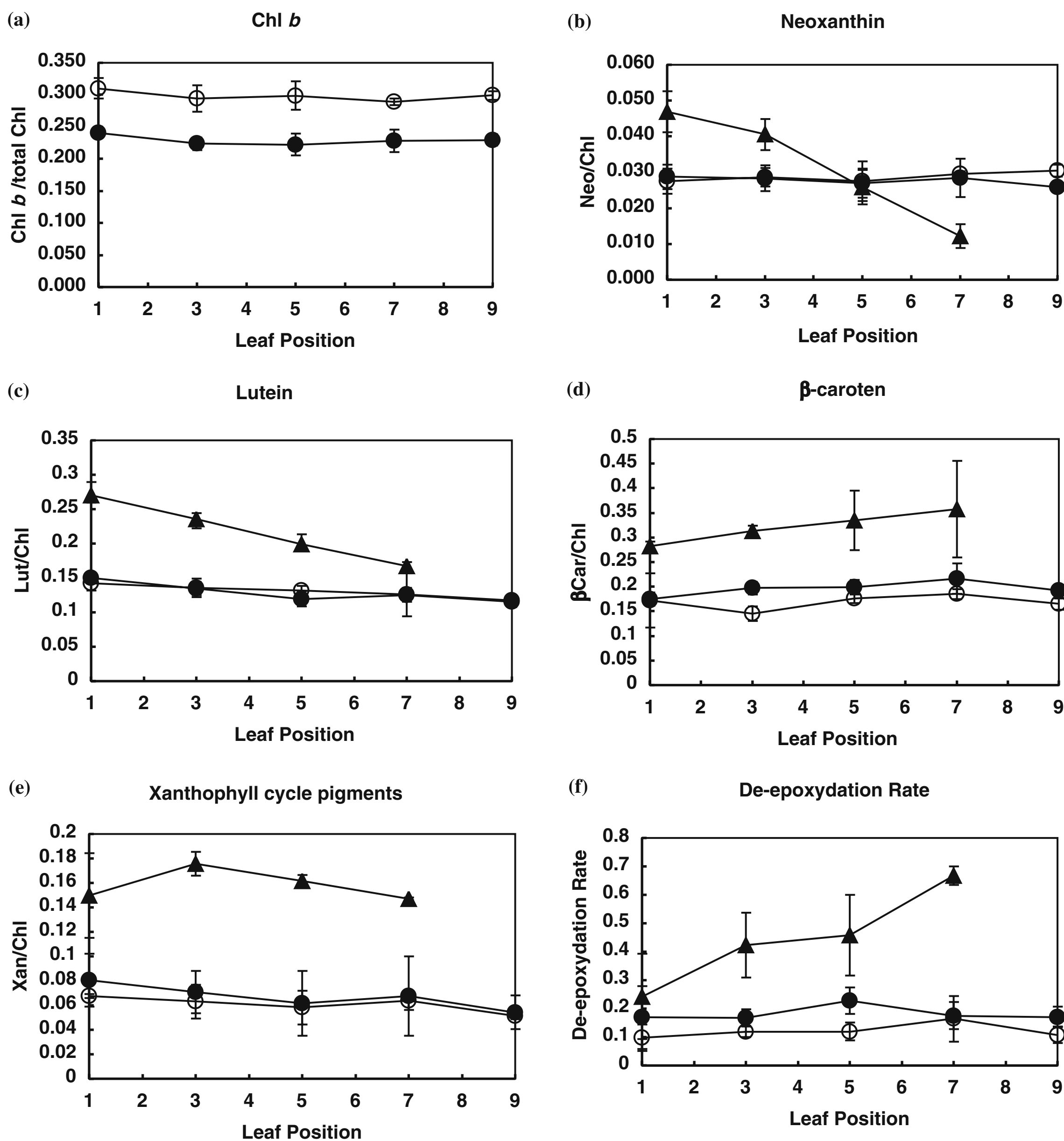


Figure 4. Chl *b* and carotenoid contents in HL-acclimated WT (closed circles), YK1-10 (open circles) and *ch1-1* (closed triangles) in leaves of different ages. (a) Chl *b*, (b) neoxanthin, (c) lutein, (d) β-carotene and (e) xanthophyll cycle pigments (total of violaxanthin, antheraxanthin and zeaxanthin contents) were expressed relative to total Chl contents (mol/mol). (f) The de-epoxydation rates were calculated as (0.5 × antheraxanthin + zeaxanthin)/(total xanthophylls cycle pigments). All the lines were plotted against the leaf position (the number of the leaf whorl) counting from the top. $n = 3$.

steady-state levels of *Lhcb1* mRNA were also correlated with light intensities (see Figure 1c). These data indicate that the correlation between mRNA levels and light intensity is not sufficient to answer the question whether the levels of *CAO* mRNA controls Chl *a*/*b* ratios in light acclimation. Therefore, we examined the Chl *a*/*b* ratios in transgenic plants overexpressing *CAO*. These analyses revealed that Chl *a*/*b* ratios were correlated with *CAO* mRNA levels in a manner that was independent of light intensity. We found that Chl *a*/*b* ratios in *CAO*-overexpression transgenic

plants were always lower than that in WT even in the highest light intensity (1200 μmol m^{-2} s^{-1}) used in this study (Figure 2c). These results unequivocally demonstrate that expression of *CAO* plays a predominant role in determining the Chl *a*/*b* ratios. Pattanyak (2005) et al. also came to the same conclusion in this point using tobacco *CAO*-overexpression transgenic plants, independently from our group. However, their plants produced larger amounts of chlorophylls, which we have never observed in our plants (data not shown). Thus, *A. thaliana* and tobacco must have different systems in coordination of Chl synthesis and Chl *a*/*b* ratios.

In addition to the Chl *a* to Chl *b* reaction catalyzed by CAO, the reverse reaction, Chl *b* to Chl *a* conversion may possibly contribute to the regulation of Chl *a*/*b* ratios. However, judging from the results obtained with the *CAO*-overexpression plants used in this study (Figure 2c), the contribution of the Chl *b* to Chl *a* activity may be less significant than that of the CAO-catalyzing Chl *a* to Chl *b* reaction. As suggested by Scheumann et al. (1999), the Chl *b* to Chl *a* reaction may play an important role during leaf senescence.

The results in this study indicated that *CAO* gene expression plays a predominant role in the control of Chl *a*/*b* ratio; at least within a range of normal Chl *a*/*b* ratios from approximately 2.5–4. We rarely obtained a *CAO*-overexpression line that showed Chl *a*/*b* ratios less than 2.5 (data not shown), although some of the transgenic lines showed a several fold increase in their *CAO* mRNA levels compared to those in WT (see Figure 2a); thereby suggesting that there is another mechanism that may limit Chl *b* biosynthesis. We found that the N-terminal domain of CAO, spanning approximately 150 residues, is essential in keeping the Chl *a*/*b* ratios within the normal range (Yamasato et al. 2005). Below this range where the Chl *a*/*b* ratio is less than 2.5, post-transcriptional control of CAO accumulation plays a crucial role in the determination of Chl *a*/*b* ratios.

A distinct set of LHC proteins are affected by CAO-*overexpression*

Is the accumulation of LHC dependent on the biosynthesis of Chl *b*, or on other factor(s) associated with light intensities? Our results on the levels of 10 LHC members demonstrated that they are classified into three groups according to their dependency on the Chl *b* levels in terms of respective levels of accumulation. Lhcb1, Lhcb3 and Lhcb6 are categorized into the first group and their accumulation is highly correlated with the Chl *a*/*b* ratios (summarized in Table 1). The accumulation of these proteins was significantly decreased in a Chl-*b* less mutant, *ch1-1*, and increased in the *CAO*-overexpression YK1-10 line (Figure 3); indicating that Chl *b* biosynthesis is involved in the control of these proteins. Although the difference in the Lhcb3 protein level between YK1-10 and WT was rather small in comparison to that of Lhcb1 levels, we confirmed this difference with repeated experiments and, therefore, substantiated its significance. Lhcb2, Lhca1 and Lhca4 belong to the second group of LHC members (Figures 3a, b and Table 1). Although they require Chl *b* for their stability, their accumulation was not increased by *CAO*-overexpression (Figures 3a and b). The mechanism regulating the accumulation of this class of proteins is not clear and it may be influenced by another factor(s) such as abundance of their mRNA, which may limit the over-accumulation of LHC proteins. It has been shown that mRNA for most Lhc genes were down-regulated with increasing light intensities (Kimura et al. 2003). The third group includes Lhcb4, Lhcb5, Lhca2 and Lhca3 and their accumulation is totally independent of Chl *b* biosynthesis (Figures 3a, b and Table 1). Pattanayak et al. described in their report that they did not observe change in LHC contents in their CAO-overexpression transgenic plants (Pattanayak et al. 2005). However, judged from their photo-images of immunoblotting, at least, accumulation of Lhcb1 seemed to have increased (Pattanayak et al. 2005). Chl-*b* dependent LHC regulatory mechanism is likely to be a common mechanism among plants.

Changes in a particular LHC protein can be a consequence of changes in another LHC protein. It was the case with Lhcb1 in an Lhcb2 antisense plant (Andersson et al. 2003; Ruban et al. 2003). In these plants, suppression of *Lhcb2* mRNA expression caused reduction in the Lhcb1 level. Thus, it is possible that not all LHC proteins that we categorized into the group 1 or 2 are Chl-*b* dependent, but area dependent on another LHC protein that is truly Chl-*b* dependent.

Table 1. The summary of immunoblotting data presented in Figure 3b and proposed LHC categories

Plant Light conditions Chl *b* availability	*ch1-1* HL none	*ch1-1* LL none	WT HL low	WT LL high	YK LL high	YK HL high	Chl *b* requirement for accumulation[d]	Regulation by Chl *b* biosynthesis[e]
Group 1[a]								
Lhcb1 (Lhcb3, Lhcb6)	+	+ + +	+ + + + + +	+ + + + + + + + + + +	+ + + + + + + + + + +	+ + + + + + + + + +	Yes	Yes
Group 2[b]								
Lhca1 (Lhcb2, Lhca4)	+	+ + +	+ + + + +	+ + +	+ + + +	+ + + + +	Yes	No
Group 3[c]								
Lhca3 (Lhcb4, Lhcb5, Lhca2)	+	+	+	+	+	+	No	No

Based on the data presented in Figure 3b, we proposed that LHC proteins can be categorized into 3 groups according to their dependency on Chl *b* biosynthesis.; [a] Proteins that were decreased in *ch1-1* and increased in YK1-10 under HL were categorized into the group 1; [b] Proteins that were decreased in *ch1-1* and not increased in YK1-10 under HL were categorized into the group 2.; [c] Group 3 includes proteins whose levels were not altered by availability of Chl *b*. One LHC protein shown in bold letters was chosen from each proposed group and its immunoblotting data was shown in this table in a simplified format. One '+' indicates the protein level found in *ch1-1* under HL conditions for each protein. The number of '+' indicates the fold increase in the protein level under the respective light conditions in each plant.; [d] Chl *b* requirement for accumulation the protein was judged by the protein level in *ch1-1*. If the protein level in ch1-1was substantially lower than that in WT, we considered that they require Chl *b* for accumulation.; [e] If the protein level in YK1-10 was higher than WT under HL, we considered that Chl *b* availability is participated in the regulation of the relevant LHC protein. For proteins in group 2 and 3, transcription of Lhc genes may play a major role in control of the protein levels. Degradation and/or vesicle transport system of LHC proteins may also play essential roles in controlling proteins of all groups.

Nevertheless, the classification criteria described above is consistent with the current knowledge on protein topology in PSs (Yakushevska et al. 2001; Ben-Shem et al. 2003; Yakushevska et al. 2003). In PSI, Lhca2 and Lhca3 form a heterodimer and both of them are categorized in the third group. Lhca1 and Lhca4 form a different heterodimer (Rupprecht et al. 2000; Schmid et al. 2002; Ben-Shem et al. 2003) and are categorized in the second group as described above. Lhcb1, Lhcb2 and Lhcb3 form heteromeric trimers surrounding PSII, and Lhcb6 links some of the trimers that are bound to the rest of PSII in a moderate strength (Yakushevska et al. 2001, 2003). It appears that plants control the accumulation of LHC proteins that form small units within PSs through the regulation of Chl *b* biosynthesis.

Such a regulatory system would have an advantage in preventing the accumulation of free Chls. In this system, LHC apoproteins belonging to the first group are excessively synthesized (Flachmann and Kühlbrandt 1995; Flachmann 1997) and if they fail to bind Chl *b*, they do not accumulate in cells (Darr et al. 1986; Harrison et al. 1993; Krol et al. 1995; Bossmann et al. 1997). This would enable the binding of all Chl *b* pigments to proteins, which may then minimize the risk of the accumulation of free Chls that are capable of generateing toxic singlet oxygen species. At the same time, Chl-*b* independent accumulation of the LHC proteins belonging to the third group may ensure the minimum size of the antenna complexes and provide the docking sites for the first group of LHC proteins to PSs.

Another interesting observation with LHC accumulation in *CAO*-overexpression plants is that the level of Lhcb5 was significantly increased in *ch1-1* under HL. A similar observation was reported with an Lhcb2 antisense line (Ruban et al. 2003), in which the levels of Lhcb1 and Lhcb2 decreased significantly, while the level of Lhcb5 increased several-fold. In this antisense line, Lhcb5 formed unusual LHC trimers with Lhcb3 and partly compensated the light-harvesting role played by Lhcb1 and Lhcb2. Our finding that the Lhcb5 levels increased in *ch1-1*, especially under HL, supports a hypothesis by Ruban et al., (2003) which stated that an unknown regulatory mechanism increases the expression of *Lhcb5* when the

major LHC proteins are depleted by certain reasons (Ruban et al. 2003).

The effects of Chl *b* deficiency on LHC accumulation has been studied extensively with a barley Chl *b*-less mutant, *chlorina-f2*. Harrison et al. (1993) found that the levels of almost all of the LHC proteins decreased in this mutant, except for that of a 25-kDa LHC protein which might correspond to Lhcb3 or Lhcb5. Consistent to our data, Bossmann et al. (1997) also reported that Lhcb1, Lhcb6 and Lhca4 were degraded in this mutant. On the other hand, Krol et al. (1995) reported that Lhcb4 instead of Lhca4 was degraded in the same mutant. It is possible that this discrepancy is due to differences in growth conditions for the plants. Alternatively, this difference might be due to the specificity of antibodies that were used for Lhcb4 and Lhca4. With exception for this discrepancy, previous observations with *chlorina-f2* are in agreement with the results in this study, indicating that the regulatory mechanism for LHC accumulation involving Chl *b* synthesis is common in higher plants.

The mechanism for the removal of LHC proteins that fail to bind Chl *b* has not yet been elucidated. It has been proposed that regulatory proteolysis (Lindahl et al. 1995) and/or a regulatory vesicle transport system (Hoober and Eggink 1999; Park et al. 1999; Hoober and Eggink 2001) are involved in removal of such LHC proteins from thylakoid membranes. In *ch1-1*, the disappearance of Lhcb2, Lhcb3, Lhca1 and Lhca4 were significant only under HL, indicating that the proteolytic and/or transport activities are much greater under stronger light intensities. It is possible that the machineries of such LHC removal activity have a different affinity to each LHC protein and that it does not recognize Lhcb4, Lhcb5, Lhca2 and Lhca3 at all. Since Chl *a* and Chl *b* seemed to have their fixed binding sites in the LHC apoproteins (Liu et al. 2004), the failure of LHC proteins to bind Chl *b* may induce specific conformational changes in the LHC structure that would be easily recognized by the proteases and/or the transport system.

Decrease in β-carotene levels in a CAO-*overexpression line*

We found that *CAO*-overexpression resulted in decreased accumulation of β-carotene by 25% at maximum, while it was significantly increased in *ch1-1* (Figure 4d). It was consistent with the observation that β-carotene was mainly bound to the core complexes (Lee and Thornber 1995). Thus, this result indicated that the proportion of LHC proteins within PSs was reduced in *ch1-1*, and was increased in YK1-10. The levels of the other carotenoid species were not significantly different between WT and YK1-10. This observation is in part explainable by the fact that part of the hydroxylated carotenoids are not bound to proteins and are present in thylakoid membranes (Tardy and Havaux 1997), while most of β-carotene is most likely bound to proteins. Therefore, only the levels of β-carotene may have reflected the proportion of the core complexes within PSs. However, since usually unbound fraction of neoxanthin pigments are believed to be low, another explanation is also possible, such as substitution of neoxanthin binding sites by other xanthophylls, as it seemed to happen in a neoxanthin-less mutant (Pogson et al. 1998).

The de-epoxydation rates were significantly higher in older leaves of *ch1-1* (see Figure 4f). It is possible that this rate change may be a result of the reduced levels of LHCII proteins in *ch1-1* (see Figure 3). Since LHCII are involved in the thermal dissipation of excessive energy (Elrad et al. 2002; Liu et al. 2004) and state transition (Andersson et al. 2003), a decrease in LHCII in *ch1-1* is likely to induce the acidification of the luminal space that may lead to increased de-epoxydatoin rates (Niyogi 1999). Conversely, we found a slight decrease in the de-epoxydation rates in YK1-10, which may have resulted from an increase in LHCII proteins in *CAO*-overexpression plants (see Figure 3).

Conclusion

We found that the overexpression of *CAO* suppressed the changes in Chl *a*/*b* ratios and lead to an increase of a distinct set of LHC proteins under HL. These results demonstrate that the expression of *CAO* controls the Chl *a*/*b* ratio and is involved in the regulatory mechanism of LHC accumulation upon light acclimation of higher plants. Collectively, our data support the hypothesis that several mechanisms exist for the control of LHC protein accumulation in plants.

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