Chlorophyll antenna size adjustments by irradiance in *Dunaliella salina* involve coordinate regulation of chlorophyll *a* oxygenase (*CAO*) and *Lhcb* gene expression

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Abstract

To elucidate the mechanism of irradiance-dependent adjustments in the chlorophyll antenna size of photosynthesis, we addressed the regulation of expression of genes encoding a variety of chlorophyll biosynthesis enzymes and that of the *Lhcb* genes in the model organism *Dunaliella salina.* Among the chlorophyll biosynthesis enzymes tested, only the chlorophyll *a* oxygenase (*CAO*) gene responded to changes in the level of irradiance with substantial mRNA level and kinetics of change that were similar to those of the *Lhcb* genes. Evidence is presented for the operation of a cytosolic signal transduction pathway for the rapid (order of minutes) regulation of both *CAO* and *Lhcb* gene expression by irradiance. Inhibitor studies and transient activation of Ca^{2+}-dependent kinase suggested phopholipase-C activation to Ca^{2+} release, and activation of a specific Ca^{2+}/CaM-dependent protein kinase in this cytosolic signal transduction pathway. The redox-state of the plastoquinone pool also serves to regulate *CAO* and *Lhcb* gene expression on a slower time scale (hours) and probably serves as a plastidic-origin signal that acts coordinately with the cytosolic signal transduction pathway. It is proposed that irradiance-dependent adjustments in the chlorophyll antenna size occur by coordinate regulation of *CAO* and *Lhcb* gene expression via two distinct signal transduction pathways in photosynthetic organisms.

Introduction

Chlorophyll (Chl)-protein complexes are major components of the photosynthetic apparatus serving to absorb light and to transfer excitation energy to photochemical reaction centers (Thornber, 1979). The amount and composition of the Chl-protein complexes is variable in thylakoid membranes, making for a variable light-harvesting Chl antenna size. This variability depends mainly on the incident irradiance during plant growth. Photosynthetic organisms acclimate to the level of irradiance (photoacclimation) by adjusting the size of the Chl antenna associated with each photosystem (Anderson, 1986; Melis, 1991). When plants are grown under low light (LL) intensity, the photosystems contain relatively high amounts of Chl *b,* have a large Chl *a*-*b* light-harvesting complex (LHC) and a high LHC to PS-core ratio. When plants are grown high light (HL) intensity, the photosystems contains relatively low amounts of Chl *b,* have a small LHC antenna and a low LHC to PS-core ratio (Björkman et al., 1972; Leong and Anderson, 1984; Larsson et al., 1987).

Irradiance-dependent adjustments of the Chl antenna size are a dynamic compensation response (Melis, 1996; 1998). When HL-acclimated cells of *D. salina* were shifted to LL-conditions, enlargement in the Chl antenna size occurred promptly with a concomitant increase in cellular Chl, LHC apoproteins and a decrease in Chl *a*/Chl *b* ratio as Chl *b* accumulated (Webb and Melis, 1995; Neidhardt et al., 1998). Increase in cellular Chl content is accompanied by parallel changes in the abundance of LHC apoproteins such that normally no excess pigment is produced without the corresponding binding protein and *vice versa*. Furthermore, Chl *b*

biosynthesis is essential for the assembly and function of most LHC proteins (Peter and Thornber, 1991; Harrison and Melis, 1992). Thus, the mechanism of chloroplast photoacclimation might involve regulation of Chl *b* biosynthesis in parallel to that of the LHC (Björkman et al., 1972; Masuda et al., 2002). Recently, a gene encoding the Chl *a* oxygenase (*CAO*), which catalyses the conversion of Chl *a* to Chl *b,* has been cloned from *C. reinhardtii* (Tanaka et al. 1998). Work from this laboratory (Masuda et al., 2002) showed that, following a HL→LL shift of *D. salina* cultures, rapid induction of both *CAO* and *Lhcb* transcripts occurred within 1.5 h, coincident with a period of rapid LHC apoprotein accumulation (LaRoche et al., 1991; Webb and Melis, 1995). These results indicated that increase in the Chl antenna size occurs by an irradiance-dependent coordinate induction of *Lhcb* and *CAO* gene expression, and of Chl biosynthesis.

The regulation of gene expression in response to irradiance is not well understood. It must involve sensing of the level of irradiance (light intensity), conversion of the electromagnetic signal to biochemical information, and transduction of this information to regulatory elements responsible for gene expression. At present, most available information about light signal transduction is limited to phytochrome-mediated responses. Our understanding of the signal transduction pathway(s) for other light responses remains relatively poor, in spite of a substantial body of physiological and biochemical literature on the photoacclimation of the photosynthetic apparatus (Anderson, 1986; Melis, 1998). Green algae have proven to be a good model systems in related studies as they generally have a greater capacity for photoacclimation than higher plants, showing significantly variable Chl and LHC contents in the thylakoid membrane (Sukenik et al., 1987; Tanaka and Melis, 1997; Nishigaki et al., 2000). Green algae lack phytochrome and phytochrome-mediated responses but they exhibit blue-light photoresponses.

Two distinct light-responsive signal cascade events occur in plastids and are proposed to control nuclear gene expression in green algae. One is proposed to involve regulation via Chl intermediates and functions as a dark/light switch for nuclear gene expression (Johanningmeier and Howell, 1984; Johanningmeier, 1988; Kropat et al., 1997, 2000). The other is thought to operate via the redox state of the plastoquinone pool for the acclimation of the chloroplast thylakoids to the level of irradiance (Escoubas et al., 1995; Huner et al., 1998). In this work, the regulation of expression of Chl biosynthesis enzymes (Beale, 1999) in relation to that of the *Lhcb* genes was investigated. It is shown that, among several different Chl biosynthesis genes, expression of the *CAO* gene is specifically regulated in co-ordination with that of the *Lhcb* genes. Biochemical and pharmacological analyses suggested co-ordination of *CAO* and *Lhcb* gene expression in response to irradiance. Evidence is presented for the operation of a fast (order of minutes) cytosolic signal transduction pathway in the cell, which involves phopholipase-C (PLC), and a specific Ca^{2+}/CaM-dependent protein kinase for a rapid irradiance-dependent change in the level of both *CAO* and *Lhcb* mRNA in *D. salina.* A slower (order of hours) regulation of expression of *CAO* and *Lhcb* genes in response to irradiance occurs via the redox state of the plastoquinone pool in the chloroplast of the green algae. The two signaling pathways may serve different functions in the acclimating photosynthetic apparatus.

Materials and methods

Cell Growth Conditions

The unicellular green alga *Dunaliella salina* Teod. (UTEX collection; Starr, 1978) was grown photoautotrophically in an artificial hypersaline medium (Pick et al., 1986) in the presence of 25

mM $NaHCO_3$ as a supplemental inorganic carbon source. Cells were grown in flat bottles (3 cm optical path length) at 30°C under continuous illumination at 50 (low light, LL), 300, 700 or 2,200 μmol photons m^{-2} s^{-1} (high light, HL). For light-shift experiments, LL-grown or HL-grown cells were transferred to HL- or LL-conditions, respectively, at the late-exponential growth phase with or without the addition of artificially added compounds. To prevent superimposed diurnal variations in the level of *Lhcb* mRNA, cells were grown under continuous illumination.

Fine biochemical and pharmacological compounds were purchased from Sigma and added to the growth medium from concentrated stock solutions. Stock solutions of actynomycin D (75 mg/mL), DBMIB (10 mM), U73122 (1 mM), U73343 (1 mM), okadaic acid (100 μM), microcystin LR (1 mM) and staurosporin (1 mM) were prepared in dimethyl sulfoxide (DMSO). Stock solutions of cycloheximide (50 mg/mL) and trifluoperazine (10 mM) were prepared in H_2O, chloramphenicol (50 mg/mL) and DCMU (10 mM) were prepared in ethanol. Control cultures were supplemented with equal concentrations of the solvents that were used in the preparation of the stock solutions.

Determination of Cellular Chlorophyll

For Chl measurements, cells were extracted in 80% acetone and debris were removed by centrifugation at 10,000*g* for 5 min. The absorbance of the supernatant at 710 nm, 663 nm and 645 nm was measured with a Shimadzu UV-160U spectrophotometer. The Chl *(a* and *b)* concentration of the samples was determined according to Arnon (1949), with equations corrected as in Melis et al. (1987). The number of cells per ml of suspension was counted using the improved Neubauer ultraplane and an Olympus BH-2 light microscope with an amplification of 200×.

RNA Isolation and Northern Hybridisations

Thirty to fifty millilitre of cell culture (approximately 2×10^6 cells/mL) were harvested by centrifugation and the total RNA was isolated using the RNeasy Mini Kit (Qiagen, Carlsbad, CA) according to manufacturer's instructions. Fifteen micrograms of RNA/lane were fractionated by electrophoresis through 1% agarose/formaldehyde gels and then transferred to a nylon membrane. A 1-kb *Eco*RI fragment containing a cDNA of *Lhcb* gene (*pDTcab1*) cloned from *D. tertiolecta* (accession No. M35860), a 1.9-kb *Xho*I-*Eco*RI fragment containing a *CAO* cDNA cloned from *D. salina* (accession No. AB021312) and a 0.4-kb PCR fragment containing an *ACT* cDNA cloned from *D. salina* (accession No. AF163669) were used as *Lhcb, CAO* and *ACT* probes, respectively. Since genes from *D. salina* generally show high homology with the respective genes of *Chlamydomonas reinhardtii*, probes for the other Chl biosynthetic enzymes were obtained from cDNA of *C. reinhardtii*. A 0.3-kb fragment containing *HEMA* cDNA (accession No. AV642373), a 0.4-kb fragment of *GSA* cDNA (accession No. BE237905) and a 0.4-kb fragment containing *CHLG* cDNA (accession No. AV623758) were obtained by RT-PCR. Probes for three subunits of Mg-chelatase (*CHLI*, *CHLD* and *CHLH*) were obtained by RT-PCR with degenerate primers designed from invariant amino acid sequences in each subunit (Willows and Beale, 1998). For *Lhcb*, *CAO, ACT, CHLI*, *CHLD* and *CHLH* probes, hybridizations were carried out at 65˚C for 16 h, and the membranes were washed twice with 2×SSC/0.1% SDS at 65˚C for 15 min, and twice with 0.2×SSC/0.1% SDS at 65˚C for 15 min. For other probes, hybridization and washing was carried out at 55˚C. For the detection of *CAO* and *Lhcb* mRNA, Northern blots were usually probed with *CAO* and *Lhcb* simultaneously, and these membranes were re-probed with *ACT* after stripping. Relative amounts of mRNA were

estimated by densitometric scanning of autoradiograms. Peak areas from the densitometric scans were used to calculate the relative abundance to the level of *ACT* for each sample.

SDS-PAGE and Western Blot Analysis

Total proteins were extracted with solubilization buffer containing 250 mM Tris-HCl (pH 6.8), 20% glycerol, 7% SDS. Forty micrograms of total protein were separated electrophoretically in a gel containing 12.5% acrylamide (Laemmli, 1970) at a constant current of 9 mA for 16 h. Electrophoretic transfer of the SDS-PAGE resolved proteins onto nitrocellulose was carried out for 3-5 h at a constant current of 800 mA, in transfer buffer containing 50 mM Tris, 380 mM glycine (pH 8.5), 20% methanol and 1% SDS. Identification of proteins was accomplished with specific antibodies raised in rabbit against *Synochococcus* GSA (Grimm et al., 1991), Soybean CHLI (Nakayama et al., 1998), *Plectonema boryanum* CHLD, and the LHC-II apoproteins (Harrison and Melis, 1992). Cross-reaction with the antibodies was visualized by the enhanced chemiluminescence (ECL) method.

Nuclear Run-on Transcription Assays

Nuclei isolation and transcription *in vitro* were performed as described by Keller et al. (1995). The pellet containing intact nuclei was resuspended in 2.5% (w/v) Ficoll, 0.5 M sorbitol, 0.008% spermidine, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5), 50% glycerol and 1 mM dithiothreitol, was frozen in liquid nitrogen and stored at -80°C until use. The yield and intactness of isolated nuclei were determined by counting 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei with a fluorescence microscope. Approximately 5×10^8 nuclei were incubated in the reaction mixture for 90 min at 30°C, and radioactively labelled RNA was extracted with RNeasy mini kit (Qiagen). The resultant ^{32}P-labeled transcripts were used as probes on nylon membranes containing as excess of selected target DNA. Membranes were prepared by slot blotting 4 μg of denatured plasmid DNA containing the *Lhcb*, *CAO* genes, the 25S, 5.8S and 18S rRNA of *C. reinhardtii* or pBluescript II as a nonspecific control. Hybridization and washing was carried out at 68°C. Relative amounts of mRNA were estimated by densitometric scanning of autoradiograms. Peak areas from the densitometric scans were used to calculate the relative abundance of each mRNA with respect to the level of rRNA in each sample.

Assay of CDPK

Cells were harvested by centrifugation at 5,000*g* for 1 min at 4°C. Pellets were resuspended in 1 mL of extraction buffer containing 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 2 mM EGTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin A and 1 mM phenylmethylsulfonylfluoride (PMSF). Cells were broken by sonication in a Branson 200 Cell Disruptor operated at 4 C for 30 s at a power output of 3 and a 50% duty cycle. Unbroken cells and starch grains were removed by centrifugation at 15,000*g* for 15 min at 4°C. The assay of CDPK was carried out at 30°C with SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase Assay System (Promega, Madison, WI). CDPK activity was expressed as the activity in the presence of Ca^{2+} minus the activity in the presence of EGTA. In-gel protein kinase assay in polyacrylamide gel was done according to the method of Yuasa et al. (1995). Fifteen micrograms of extracted protein was separated in a 10% polyacrylamide gel containing 1 mg/mL dephospholylated casein (Sigma) as the substrate. Protein concentration was determined by the method of Bradford (1976) using BSA as standard.

Results

Irradiance-Dependent Expression of Genes in D. salina

The irradiance-dependent expression of Chl biosynthesis and LHC-II genes of *D. salina* was investigated upon cell growth under different light intensities. Figure 1A shows steady state mRNA levels of several key genes encoding Chl biosynthesis enzymes, i.e., glutamyl-tRNA reductase (*HEMA*), glutamate 1-semialdehyde aminotransferase (*GSA*), three subunits of magnesium chelatase (*CHLI*, *CHLD*, *CHLH*), chlorophyll *a* synthetase (*CHLG*), and chlorophyll *a* oxygenase (*CAO*). Among the Chl biosynthesis genes tested, only *CAO* showed a declining steady state level of mRNA with increasing growth irradiance, a property similar to that of the *Lhcb* gene. Thus, the steady state level of *CAO* mRNA under low light (LL: 50 μmol photons m^{-2} s^{-1}) was approximately 5 times greater than that under high light (HL: 2,200 μmol photons m^{-2} s^{-1}) conditions. The mRNA level of *HEMA*, *GSA* and *CHLG* was fairly constant under all growth irradiances. Interestingly, the steady-state mRNA level of the Mg-chelatase subunits (*CHLI*, *CHLD*, and *CHLH*) increased with growth irradiance. Western blot analysis of CHLI and CHLD confirmed greater accumulation of these proteins under HL-growth conditions, whereas the level of GSA remained constant (Figure 1B). Western blot analysis with anti-LHC-II antibodies showed accumulation of four apoproteins of LHC-II under LL-growth and significant depletion of LHC-II under HL-growth conditions, consistent with previous reports from this laboratory (Webb and Melis, 1995; Tanaka and Melis, 1997).

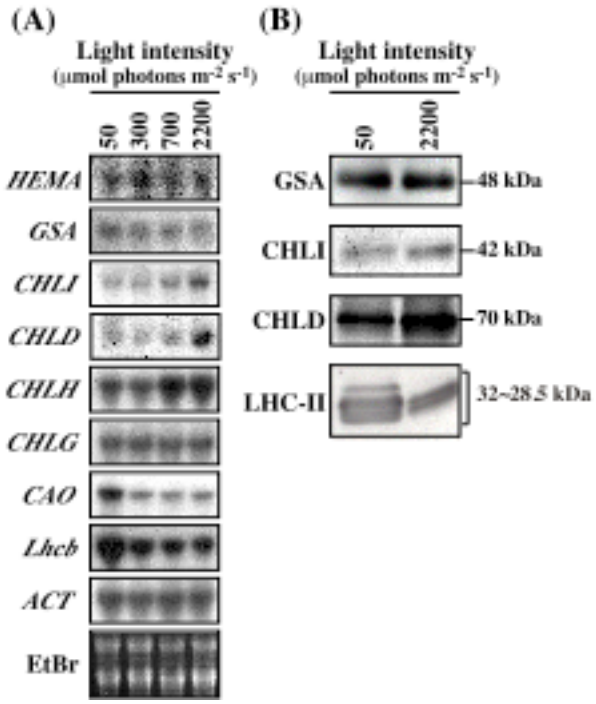

Figure 1. Steady state transcript levels of Chl biosynthesis and LHC-II genes from *D. salina* grown under continuous illumination of different intensities. (A) Northern blot analysis of seven different Chl biosynthesis enzymes, actin (*ACT*), *Lhcb* and ethidium bromide staining of rRNA. Cells were grown at 50, 300, 700, or 2200 μmol photons m^{-2} s^{-1}. 20 μg of total RNA was loaded in each lane. (B) Western blot analysis of total cell protein from LL-grown (50 μmol photons m^{-2} s^{-1}) and HL-grown (2,200 μmol photons m^{-2} s^{-1}) *D. salina.* 40 μg of total cellular protein was loaded in each lane.

To probe the molecular mechanism of photoacclimation in *D. salina,* light-shift experiments (HL→LL and LL→HL) were conducted, followed by measurement of the steady-state mRNA level for the Chl biosynthesis genes discussed in Fig. 1. Figure 2A (left panel) shows that, following a HL→LL shift, level of expression for the *CAO* and *Lhcb* genes promptly increased in an apparently coordinate manner. Steady state levels of mRNA for the *CHLI, CHLD, CHLH* genes decreased following HL→LL shift. The expression level of the other Chl biosynthesis genes *(HEMA, GSA, CHLG)* and that of actin *(ACT)* were not changed following the HL→LL shift. Fig. 2A (right panel) shows that, following a LL→HL shift, steady-state levels of *CAO* and *Lhcb* mRNA decreased promptly and substantially. Steady state levels of mRNA for the *CHLI, CHLD, CHLH* genes increased following the LL→HL shift, which is consistent with the higher levels of the Mg-chelatase subunits seen the HL growth condition (Fig. 1B). Again, the expression level of the other Chl biosynthesis genes *(HEMA, GSA, CHLG)* and that of actin *(ACT)* were not changed following the LL→HL shift.

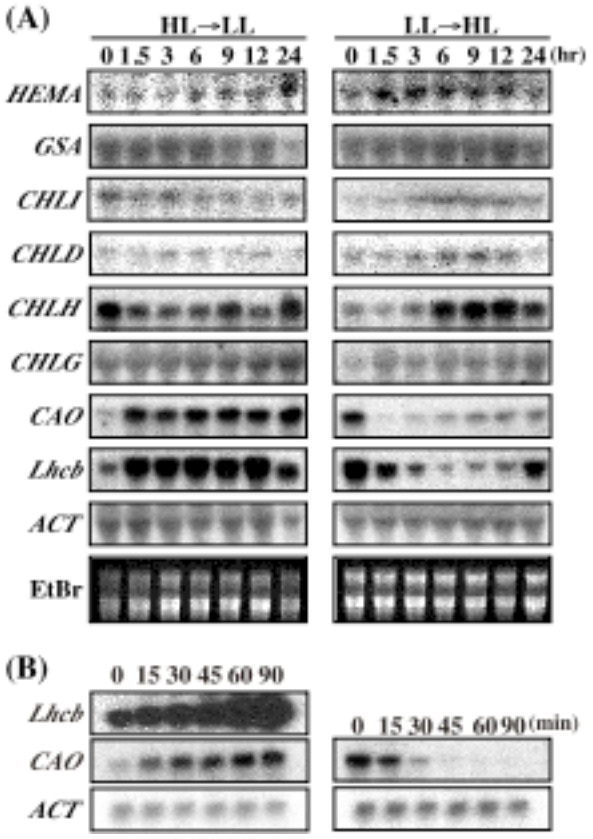

Figure 2. Kinetics of change in the level of transcripts of Chl biosynthesis and LHC-II genes following a HL→LL shift (left-side panels) or a LL→HL shift (right-side panels). (A) Northern blot analysis of total cellular RNA probed with 7 different Chl biosynthesis, actin (*ACT*) and *Lhcb* genes. Also shown is the corresponding Ethidium Bromide staining of rRNA. Cells grown under HL- or LL-conditions, were shifted at zero time to LL or HL-conditions, respectively. Aliquots were harvested for analysis at various times following the light shift, as indicated. (B) Northern blot and quantitative densitometric analyses of *CAO* and *Lhcb* mRNA following a HL→LL shift, and following a LL→HL shift. Levels of mRNA were normalized to that of *ACT* mRNA in the samples.

The kinetics of change in the level of *CAO* and *Lhcb* mRNA following a light-shift were examined in greater detail relative to actin (*ACT*), which is thought to be a constitutively

expressed structural protein in the cells. Figure 2B (left panel) shows the kinetics of change in the steady-state level of mRNA following a HL→LL shift (0-90 min). Levels of both *Lhcb* and *CAO* transcripts were induced with a half time of about 15 min. They reached a 5-fold higher steady-state level within about 90 min. Following a LL→HL shift, levels of *Lhcb* transcripts decreased with a half-time of 60 min (Fig. 2A, right panel) whereas levels of *CAO* transcripts decreased with a half-time of 15 min (Fig. 2B, right panel). Levels of actin (*ACT*) mRNA were not affected by the change in irradiance. These results clearly show that expression of the *CAO* and *Lhcb* genes is strongly regulated during photoacclimation in *D. salina.*

CAO and Lhcb Transcription Rates and mRNA Stability during Photoacclimation

Rates of *Lhcb* and *CAO* transcription were assyed from run-on transcription measurements with isolated *D. salina* nuclei. Control HL- or LL-grown cells were harvested prior to a shift in irradiance. Photoacclimating samples were harvested 1.5 h following either a HL→LL or LL→HL shift. (At 1.5 h after a shift in irradiance, levels of mRNA showed a maximal change, e.g. Fig. 2A). These run-on assays showed that transcriptional activities of *Lhcb* and *CAO* were low in HL-acclimated cells. Following a HL→LL shift, rates of transcription of both genes increased by about 5-fold within 1.5 h (Figure 3, left panels, A-C), demonstrating that induction of *Lhcb* and *CAO* mRNA following a HL→LL shift is primarily a consequence of transcriptional activation of these genes. Conversely, following a LL→HL shift, rates of transcription of both genes decreased to a very low level within 1.5 h (Figure 3, right panels, D-F).

The stability of *Lhcb* and *CAO* mRNA was assessed as a function of time under continuous HL- and LL-conditions by treatment of the cells with actinomycin D, a transcriptional inhibitor. Under our assay conditions, 75 μg/mL actinomycin D caused approximately 80% inhibition of the *in vivo* transcription rate (not shown). We found that the stability of *Lhcb* mRNA in HL was about the same as that in LL. Figure 4A shows that the half time of *Lhcb* mRNA was about 100 min, either under LL or HL steady state conditions, evidence that the level of irradiance does not affect the stability of this mRNA. Figure 4B shows that the *CAO* gene mRNA stability under LL (half time of 140 min) was greater than that under HL (~95 min). Nevertheless, these half times were considerably longer than the half time of *CAO* mRNA decrease observed upon a LL→HL shift (~15 min, Figure 2B). The results suggest that small differences in the stability of the *CAO* mRNA under LL and HL conditions are not the primary determinant of steady-state mRNA level. Rather, change in the abundance of *Lhcb* and *CAO* mRNA is primarily a consequence of regulation in the rate of gene transcription by irradiance.

Redox State of the PQ Pool and Levels of CAO and Lhcb mRNA

Changes in the level of irradiance during cell growth affect chloroplast function by altering the redox state of electron transport intermediates in the chloroplast thylakoids. These changes may constitute signal perception and may lead to a plastid-derived signal transduction pathway for the regulation of nuclear gene expression. Specifically, the redox state of the intermediate plastoquinone pool in the chloroplast thylakoids may serve in the signal transduction pathway (Durnford and Falkowski, 1997; Escoubas et al., 1995; Huner et al., 1998). Escoubas et al. (1995) showed that addition of DCMU to HL-grown *D. tertiolecta* caused a LL acclimation response. DCMU blocks electron transport from Q_A to plastoquinone (PQ) and results in the oxidation of the PQ pool, thereby mimicking the PQ redox condition that would prevail in LL. In contrast to DCMU, DBMIB induced a HL acclimation response under LL-growth conditions. This was attributed to a DBMIB inhibition of electron transport from plastohydroquinone (PQH_2) to the cytochrome *b-f* complex, resulting in accumulation of PQH_2. These studies suggested that

the redox state of the plastoquinone pool might be a sensor leading to photosynthetic apparatus acclimation to irradiance.

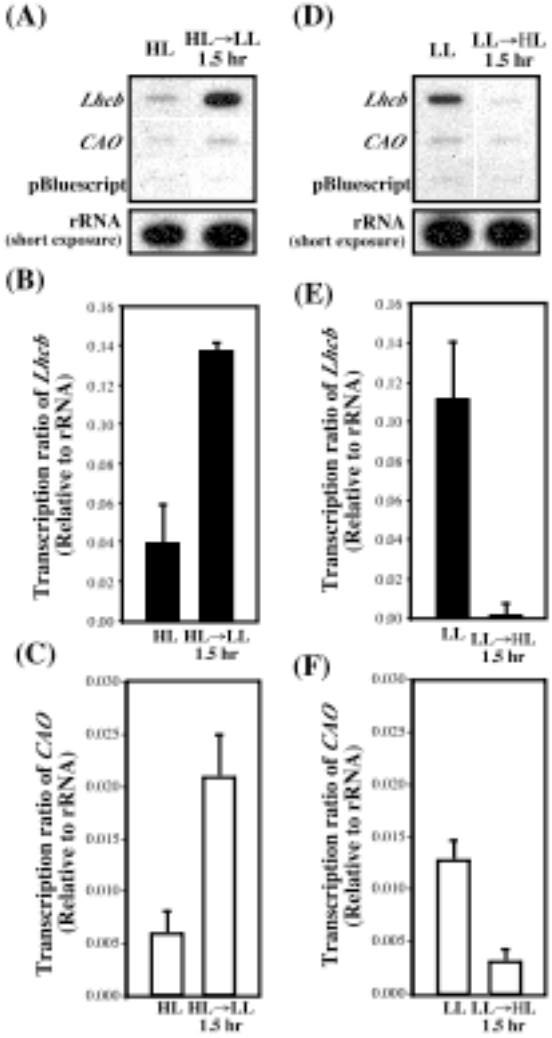

Figure 3. Run-on *Lhcb* and *CAO* gene transcription assays with isolated nuclei from *D. salina* before and after light shifts. HL- or LL-grown cells were harvested either prior to or 1.5 h after a light shift of the culture. For each data point, total RNA was purified from isolated nuclei, the *Lhcb* and *CAO* transcript levels were determined by slot-blot hybridization and normalized to rRNA after subtraction of background (pBluescript). (A) Autoradiograph of slot-blot hybridizations in HL-grown samples and samples following a HL→LL shift. (B and C) Histograms of the quantitative results for *Lhcb* and *CAO,* respectively. (D) Autoradiograph of slot-blot hybridizations in LL-grown samples and samples following a LL→HL shift. (E and F) Histogram of the quantitative results for *Lhcb* and *CAO,* respectively. Data are presented as the mean ± SD (n = 3).

To further test for the role of this signaling pathway in the Chl antenna size adjustment, we extended such studies to include the effect of DCMU and DBMIB on the level *CAO* and *Lhcb* mRNA. Figure 5 shows that addition of sublethal concentrations of DCMU (100 nM) to HL-acclimated cells and incubation for 1.5 or 8 h did not bring about a change in the levels of *CAO* and *Lhcb* mRNA. However, after 24 h incubation in the presence of DCMU, the abundance of *CAO* and *Lhcb* mRNA increased by about 50%. Similarly, Fig. 5 shows that addition of 100 nM DBMIB and incubation for 1.5 or 8 h did not bring about a change in the levels of *CAO* and *Lhcb*

mRNA, whereas after 24 h incubation with DBMIB the level of both mRNAs decreased by about 40%. Thus, there is a pronounced lag in the effect of DCMU and DMBIB on the *CAO* and *Lhcb* mRNA levels. Such lag suggests that the redox state of the PQ pool may not be the primary signal transduction pathway in the regulation of *CAO* and *Lhcb* gene transcription. Rather, it suggests the operation of a different but faster irradiance-dependent signaling pathway.

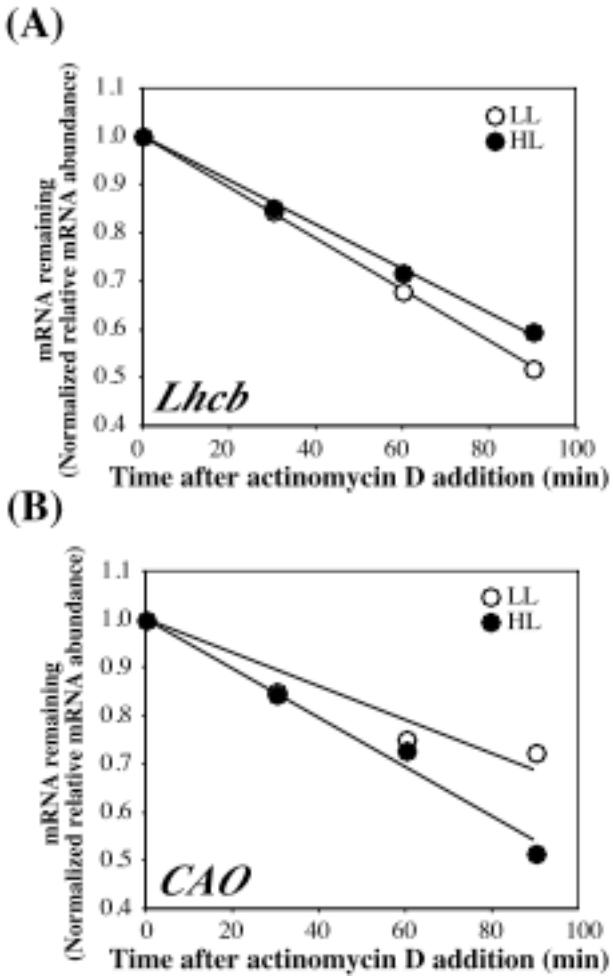

Figure 4. Stability of *Lhcb* and *CAO* mRNA in HL-grown and LL-grown cultures. (A) Comparison of the relative level of *Lhcb* mRNA and (B) *CAO* mRNA in LL-grown cells (open circles) and HL-grown cells (close circles). 75 μg/ml of the transcription inhibitor actinomycin D were added directly to the respective cultures at zero time. Total RNA was isolated at the indicated times. The level of each mRNA was measured by densitometry following a Northern blot analysis. Data were normalized to the corresponding level of *ACT* mRNA in the samples.

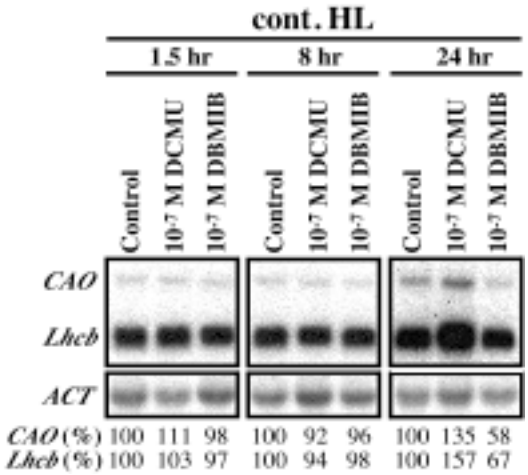

Figure 5. Effect of electron-transport inhibitors (DCMU and DBMIB) on the level of *Lhcb* and *CAO* mRNA in HL-grown *D. salina.* HL-grown cells were divided in equal aliquots and electron-transport inhibitors were added to the final concentration indicated. The effect of DCMU and DBMIB inhibition on *Lhcb* and *CAO* mRNA abundance in HL-grown *D. salina* was assayed after 1.5, 8 and 24 h incubation. Northern blot quantitative analyses of *CAO* and *Lhcb* mRNA as described in Fig. 2.

The effect of DCMU and DBMIB was investigated further upon light shift experiments conducted in the presence of these electron-transport inhibitors. Figure 6A shows that the presence of DCMU and DBMIB (concentrations of 100 nM or 1 μM) did not affect the induction of *CAO* and *Lhcb* mRNA following a HL→LL shift. Similarly, Fig. 6B shows that the presence of DCMU and DBMIB (concentrations of 100 nM or 1 μM) did not affect the down-regulation of *CAO* and *Lhcb* gene expression following a LL→HL shift. Thus, a range of concentrations of DCMU and DBMIB, from the sublethal to fully inhibitory, did not bring about a prompt change in mRNA abundance at 1.5 h following either a HL→LL or a LL→HL shift (Fig. 6). These results further suggest that the redox-state of the plastoquinone pool may not be involved in the rapid (half times of 10-40 min) *CAO* and *Lhcb* mRNA change following a change in the level of irradiance. Rather, it appears that regulation by the redox-state of the plastoquinone pool brings about changes more slowly (e.g. 24 h, Fig. 5) and may thus be responsible for a longer-term photoacclimation response in *D. salina*. It may be concluded that there is another signalling pathway, which more rapidly responds to a change in the level of irradiance in green algae, and which serves as the primary regulator of *CAO* and *Lhcb* gene expression by irradiance.

Inhibition of Protein Biosynthesis and Regulation of CAO and Lhcb mRNA

The requirement of *de novo* protein biosynthesis for an irradiance-dependent rapid change in the level of *CAO* and *Lhcb* mRNA was investigated. Cycloheximide and chloramphenicol (50 μg/mL), which are inhibitors of cytoplasmic and plastidic protein biosynthesis, respectively, were added to HL- or LL-acclimated cells immediately prior to a shift in light intensity. Cells were incubated for 1.5 h in the presence of these inhibitors under the new light regime. In the presence of cycloheximide, induction of *CAO* and *Lhcb* mRNA following a HL→LL shift was inhibited by about 70%. However, a lowering in the level of these mRNAs following a LL→HL shift was not affected by cycloheximide (data not shown). Chloramphenicol had no effect on the regulation of *CAO* and *Lhcb* mRNA level following a change in irradiance. These results suggest that *de novo* protein biosynthesis in the cytoplasm is required for the rapid increase in *CAO* and

Lhcb mRNA level following a HL→LL shift, but not so for the lowering in mRNA level following a LL→HL shift.

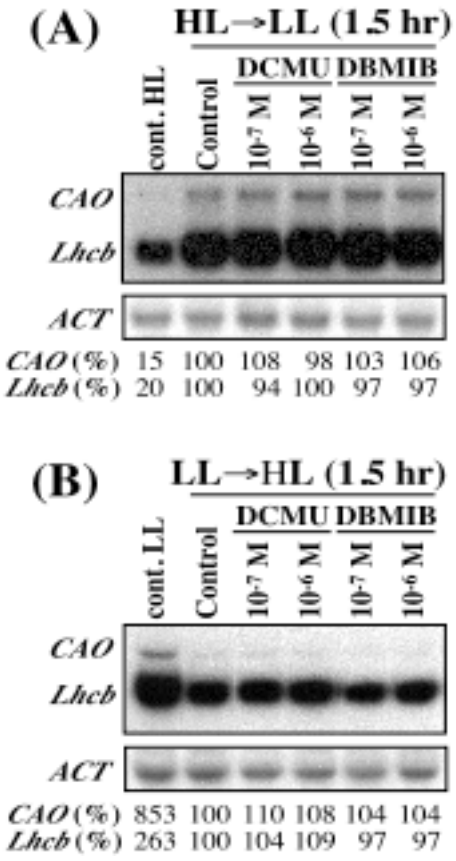

Figure 6. Effect of electron-transport inhibitors (DCMU and DBMIB) on the level of *Lhcb* and *CAO* mRNA following a HL→LL or a LL→HL shift in *D. salina.* (A) HL-grown cells were divided in five equal aliquots. Electron-transport inhibitors (DCMU and DBMIB) were added to the final concentration indicated. Cultures were immediately shifted to LL-growth conditions. The effect of DCMU or DBMIB addition on the *CAO* and *Lhcb* mRNA levels was assayed following 1.5 h incubation under LL. (B) LL-grown cells were divided in five equal aliquots. Electron-transport inhibitors were added to the final concentration indicated. Cultures were immediately shifted to HL-growth conditions. The effect of DCMU and DBMIB addition on the *CAO* and *Lhcb* mRNA levels was assayed following 1.5 h incubation under HL. The numbers below each panel show the intensity of the respective band measured upon a densitometric scan.

Phospholipase C Antagonists Inhibit Induction of CAO and Lhcb mRNA

To further explore the signal transduction pathway for an irradiance-dependent regulation of *CAO* and *Lhcb* gene expression, we employed novel pharmacological approaches. The basis of this experimentation is a work by Im and Beale (2000), who postulated activation of heterotrimeric GTP-binding regulatory proteins (G-proteins) in the induction of *GSA* by blue light in *C. reinhardtii*. Blue-light enhances the Chl biosynthetic pathway and results in 30-35% greater Chl *b* and LHC-II content, and a larger Chl antenna size for the photosystems in *C. reinhardtii* (Melis et al. 1996). Thus, blue light and heterotrimeric G-proteins may serve as key upstream

components of signal transduction pathways in the regulation of the Chl antenna size. In such a signal transduction pathway, hydrolysis of phosphatidyl-D-inositol 4,5-bisphosphate to yield D-inositol 1,4,5-triphosphate ($InsP_3$) and diacylglycerol by phospholipase C (PLC) could be the next step in the signal transduction pathway. The latter is ubiquitous in signaling cascades of plant and animal systems (Hansen et al., 1995; Munnik et al., 1998). Consistent with this model, it has been proposed that in *D. salina* the plasma-membrane-localized PLC is regulated by G-proteins (Einspahr et al., 1989). Therefore, we tested known PLC antagonists for possible interference with the induction of *CAO* and *Lhcb* following a HL→LL shift. The aminosteroid U73122 is a potent PLC antagonist that has been used to investigate the involvement of PLC in many signaling pathways (Thompson et al., 1991; Pingret et al., 1998). U73343, a less active analog of U73122, has been used as a negative control for the nonspecific effects of U73122 on PLC. Figure 7A clearly shows that U73122, in a concentration-dependent manner, significantly inhibited the induction of both *CAO* and *Lhcb* genes expression following a HL→LL shift. Figure 7B shows that the less active analog, U73343, brought about the same extent of inhibition at a much higher concentration than that required for U73122. We estimated that a 10-fold higher concentration of U73343 was needed to bring about the same extent of inhibition in *CAO* and *Lhcb* mRNA induction as the U73122 (not shown). These results clearly implicate PLC in the signal transduction pathway for the induction of *CAO* and *Lhcb* expression in *D. salina* following a HL→LL shift.

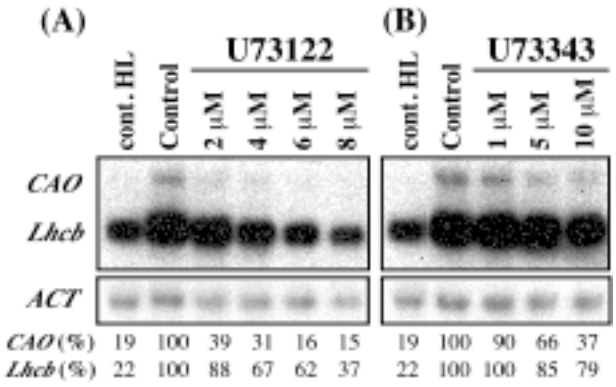

Figure 7. Effect of phospholipase-C (PLC) inhibitors U73122 and U73343 on the induction of *CAO* and *Lhcb* mRNA following a HL→LL shift (1.5 h). The indicated concentration of U73122 (A) and U73343 (B) was added to HL-grown cells immediately prior to a HL→LL shift. Following a 1.5 h incubation under LL-conditions, total RNA was isolated. Northern blots were produced and the effect of U73122 and U73343 on the *CAO* and *Lhcb* mRNA levels was assayed. The numbers below each panel show the intensity of the respective band measured upon a densitometric scan.

Calmodulin Antagonist and Protein Kinase Inhibitors Block the Induction in the Level of CAO and Lhcb mRNA Following a HL→LL Shift

In phospholipid signaling, one effect of $InsP_3$ is to bind receptors, triggering the opening of Ca^{2+} channels (Bush, 1995; Munnik et al., 1998). Einspahr et al. (1989) proposed that the plasma-membrane-localized PLC of *D. salina* is subject to Ca^{2+} regulation. One way in which Ca^{2+} is involved in signal transduction, leading to regulation of gene expression, is through interaction with the ubiquitous Ca^{2+}-binding protein calmodulin (CaM). The Ca^{2+}/CaM complex is capable of activating various protein kinases and phosphatases that in turn affect downstream signaling events. Trifluoperazine is a well-known CaM antagonist that blocks its interaction with target effector proteins (Vandonselaar et al., 1994). We found that trifluoperazine inhibits the induction

of *CAO* and *Lhcb* mRNA abundance following a HL→LL shift. Figure 8A shows that such inhibition is concentration-dependent, so that 8-10 μM trifluoperazine completely blocked induction of both *CAO* and *Lhcb* mRNA following a HL→LL shift.

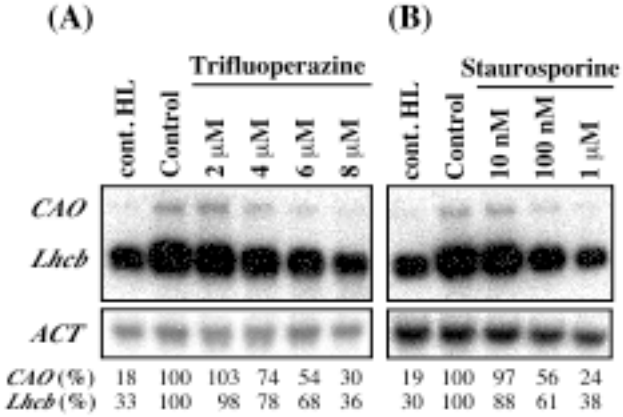

Figure 8. Effects of the calmodulin (CaM) antagonist trifluoperazine and protein kinase inhibitor staurosporine on the induction of *CAO* and *Lhcb* mRNA following a HL→LL shift (1.5 h). The indicated concentration of trifluoperazine (A) and staurosporine (B) was added to HL-grown cells immediately prior to a HL→LL shift. Following a 1.5 h incubation under LL-conditions, total RNA was isolated, Northern blots were developed, and the effect of trifluoperazine and staurosporine on the *CAO* and *Lhcb* mRNA levels was assayed. The numbers below each panel show the intensity of the respective band measured upon a densitometric scan.

Ca^{2+}/CaM is known to modulate the activity of several different Ca^{2+}/CaM-dependent protein kinases (CDPKs) such as CaM kinase II. It was previously reported that CDPKs are involved in light-dependent responses in plant cells (Lu et al., 1996). By using staurosporine, a protein kinase inhibitor, we examined whether the signal for *CAO* and *Lhcb* induction is transmitted from Ca^{2+}/CaM via a CDPK. Figure 8B shows that the induction of *CAO* and *Lhcb* mRNA abundance following a HL→LL shift was also blocked by staurosporine in a concentration-dependent manner. At 1 μM staurosporine, the induction of both *CAO* and *Lhcb* mRNA following a HL→LL shift was completely blocked, suggesting that activation of Ca^{2+}/CaM is involved in the transmittance of the light intensity signal for *CAO* and *Lhcb* induction through a Ca^{2+}/CaM-activated protein kinase.

Activation of a 54 kDa CDPK following a HL→LL Shift in D. salina

To further examine whether activation of CDPK is involved in the regulation of *CAO* and *Lhcb* expression by irradiance, we measured the effect of a HL→LL shift on the activity of CDPK in *D. salina.* This was implemented with a synthetic peptide serving as specific substrate for the CDPK reaction (Goueli et al., 1995). As shown in Fig. 9A, the level of CDPK activity was transiently induced by about 4-fold and reached a maximum at 15 min following the HL→LL shift. CDPK activity gradually declined at longer incubation times following the HL→LL shift.

In order to identify the specific CDPK affected by the HL→LL shift, we then performed an in-gel kinase activity assay with casein as a substrate (Yuasa et al., 1995). Fig. 9B shows that two protein bands, with molecular mass of 97 and 40 kDa, were detected in the presence of 2 mM EGTA (absence of Ca^{2+}). These 97 and 40 kDa bands originate from Ca^{2+}-independent protein kinase activity. Fig. 9C shows that, in the presence of 1 mM $CaCl_2$, an additional major band at 54 kDa was detected. The latter originates from Ca^{2+}-dependent CDPK activity. The activity of

the 54 kDa CDPK was considerably greater 15 min after a HL→LL shift, whereas the activities of the Ca^{2+}-independent protein kinases remained constant during this time period. These results strongly support the notion that a signal transduction process in initiated upon a HL→LL shift of the cultures and it probably involves activation of a specific CDPK (the 54 kD protein) in the induction of *CAO* and *Lhcb* mRNA levels as a function of time in LL.

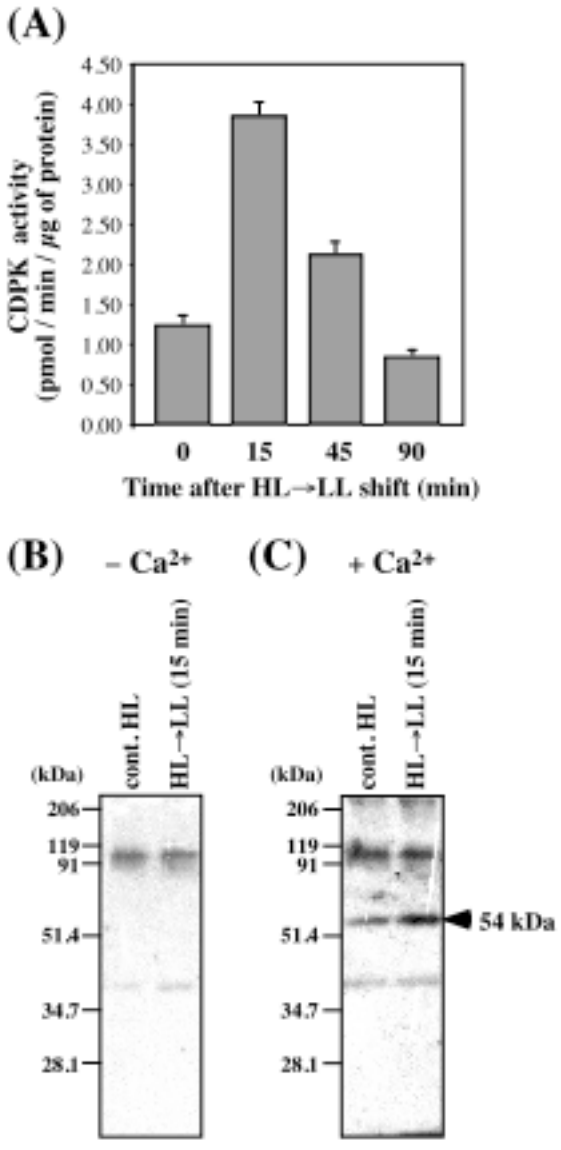

Figure 9. Activation of 54 kDa CDPK following a HL→LL shift of *D. salina*. (A) Changes in the CDPK activity following a HL→LL shift. HL-grown cells were shifted to LL at zero time, and sampled at the indicated times. Data are presented as the mean ±SD (n = 3). (B and C) In-gel kinase assays were performed in the presence of 2 mM EGTA (B) or 1 mM $CaCl_2$ (C). 15 μg of protein from samples grown under continuous HL and after 15 min incubation following a HL→LL shift were separated on 10% SDS-PAGE containing 1 mg/mL casein. CDPK activity was assayed in-gel according the protocol listed in the materials and methods section. The electrophoretic mobility of molecular weight markers is indicated.

Discussion

The green alga *D. salina* is a good model organism in which to study the acclimation of the photosynthetic apparatus to irradiance. *D. salina* can grow photoautotrophically under a broad range of light intensities and shows amplitudes of photoacclimation responses that are substantially greater than that of vascular plant chloroplasts (Melis, 1998). A significant acclimation response to the level of irradiance entails adjustment in the Chl antenna size of the photosystems. In *D. salina,* the Chl antenna size of PSII has been reported to be as small as 60 Chl molecules under HL and as large as 460 Chl molecules under LL-growth (Smith et al. 1990). It has been reported that regulation of Chl *b* biosynthesis is important in the adjustment of the Chl antenna size to irradiance (Björkman et al., 1972). Indeed, Tanaka et al. (2001) reported that overexpression of *CAO* in Arabidopsis caused enlargement of the Chl antenna size of PSII. Masuda et al. (2002) showed prompt induction for *CAO* and *Lhcb* mRNA following a HL→LL shift. In this work, biochemical and pharmacological analyses focused on the signal transduction pathway for Chl antenna size adjustment. It is shown that irradiance-dependent regulation of gene expression for *CAO* and *Lhcb* in *D. salina* employs a cytosolic signaling pathway that affects both genes in a similar fashion.

A Working Hypothesis Model for the Perception/Transduction of Irradiance Signals in D. salina

Conclusions drawn from our pharmacological and biochemical analyses are consistent with a signal transduction model for the photoacclimation of *D. salina,* schematically illustrated in Figure 10. This working hypothesis model is based on results reported in this work and is consistent with known signal transduction pathways from plant and animal cells. Such a canonical signaling pathway is proposed to involve the following cascade of reactions.

- Activation of an irradiance receptor molecule (blue light photoreceptor?).
- Heterotrimeric G-proteins are activated by the irradiance receptor molecule.
- Activated G-proteins cause a stimulation in PLC activity.
- Enhanced PLC activity causes elevation in the cytoplasmic $InsP_3$ concentration.
- Increased $InsP_3$ concentration causes in turn an increase in the cytoplasmic Ca^{2+} concentration, by triggering the release of Ca^{2+} from intracellular storage areas.
- Ca^{2+} combines with CaM. The Ca^{2+}/CaM complex activates a CDPK, which transmits the signal for *CAO* and *Lhcb* mRNA induction through a phosphorylation of a putative transfactor.

This working hypothesis is consistent with the results presented in this work and consistent with all related information in the literature. There is an increasing body of biochemical evidence to suggest that G-protein signal transduction pathways are involved in response to a variety of stimuli in plants such as hormones, light, and pathogens (Ma, 1994; Pingret et al., 1998; Plakidou-Dymock et al., 1998; Okamoto et al., 2001). Pingret et al. (1998) reported that the *Rhizobium* Nod factor signal transduction mechanism involves G-protein mediation coupled to the activation of both phosphoinositide and a Ca^{2+} secondary messenger pathway. In the light signaling pathway of vascular plants, evidence for the involvement of G-proteins, Ca^{2+}/CaM signaling and protein kinase activity is rapidly accumulating (Lam et al., 1989; Neuhaus et al.; 1993, Bowler et al., 1994; Okamoto et al., 2001). In *D. tertiolecta*, hypo-osmotic stress activates PLC and CDPK activities, suggesting involvement of $InsP_3$, increase in cytoplasmic Ca^{2+} concentration, and subsequent activation of CDPK (Einspahr et al. 1989; Pinontoan et al., 2000). In *C. reinhardtii,* induction of GSA by blue light involves heterotrimeric G-protein activation, PLC-catalyzed $InsP_3$ formation, $InsP_3$-dependent Ca^{2+} release, and activation of a downstream signaling pathway through a CDPK (Im et al., 1996; Im and Beale, 2000), very

much along the lines of the model in Fig. 10. Thus, results in the literature suggest that this cytosolic signaling pathway ubiquitously exists and functions in plant systems.

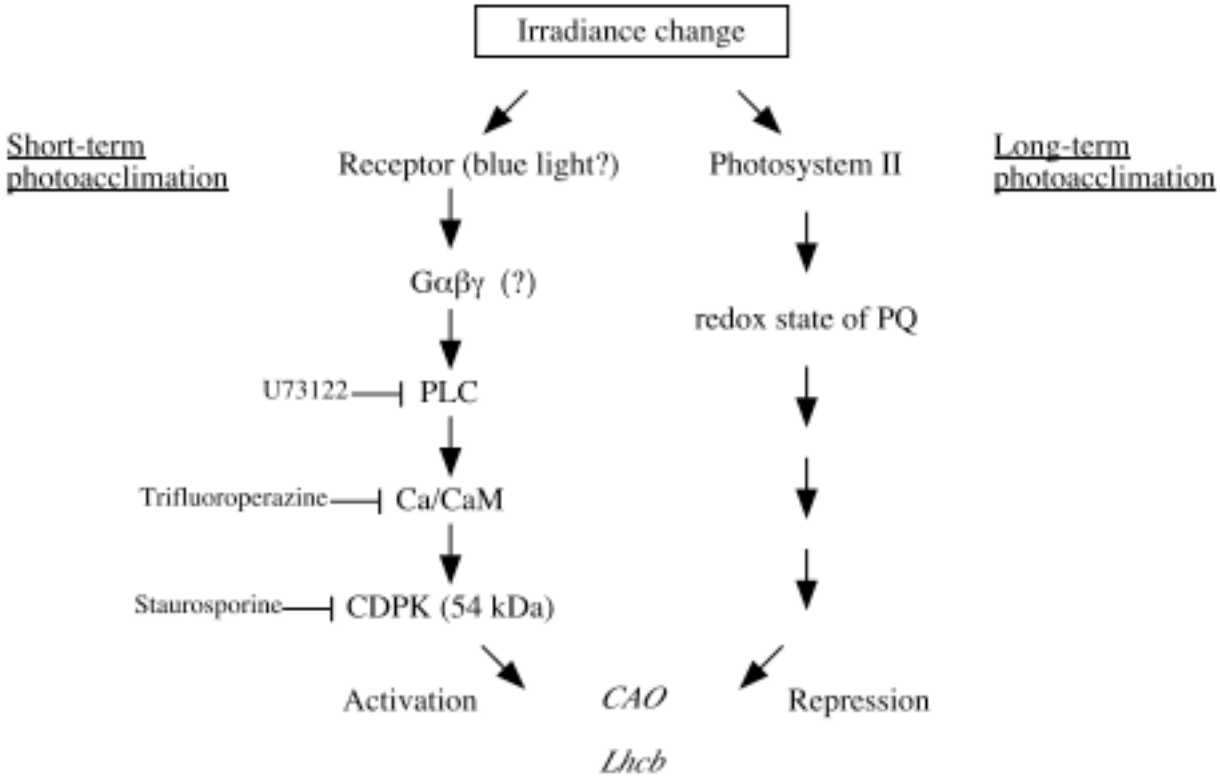

Figure 10. Working hypothesis model for a signal transduction pathway leading to regulation of *CAO* and *Lhcb* gene expression by irradiance in *D. salina*. This model is based on results presented in this work and on analogies with known signal transduction pathways in animal and plant cell systems. Gαβγ, heterotrimeric G protein; $InsP_3$, D-inositol 1,4,5-triphosphate; PIP_2, phosphatidyl-D-inositol; TF, putative transcription factor. The site of action of the various inhibitors is indicated.

In mammalian cells, G-proteins almost invariably interact with a family of structurally similar receptor proteins, which possess seven-transmembrane helices and are localized in the cell's surface (Dohlman el al., 1991). The PLC proteins are also thought to be integral to the plasma membrane, a property confirmed in *D. salina* (Einspahr et al., 1989). Thus, our working hypothesis postulates that G-proteins reversibly associate with the plasma membrane bound photoreceptors and PLC proteins to actuate the signal transduction pathway. Presently, only one gene encoding a G-protein-coupled transmembrane receptor has been cloned from a plant species, the *GCR1* gene in Arabidopsis (Plakidou-Dymock et al., 1998). Therefore, the molecular nature of the putative receptor and its function in plants and green algae remains to be elucidated.

Cooperative Regulation of the Chl Antenna Size by Two Distinct Signaling Pathways

Results in this work showed that rapid (half times of 10-30 min) irradiance-dependent changes of *CAO* and *Lhcb* mRNA level were not subject to regulation by the redox-state of the plastoquinone pool. Since cycloheximide inhibited the induction of both *CAO* and *Lhcb* mRNA following a HL→LL shift, but did not prevent the lowering of the mRNA levels following a LL→HL

shift, the mechanism of induction and lowering of *CAO* and *Lhcb* mRNA levels must be different. For the rapid induction of *CAO* and *Lhcb* mRNA following a HL→LL shift, we postulate the operation of a cytosolic signal transduction pathway in the transmittance of the light intensity signal in *D. salina*. This signaling pathway begins at the plasma membrane of the cell and may be transmitted through the cytoplasm, nucleus and chloroplast in vascular plant systems. In this respect, it is important to note that this signaling pathway accounted for the perception and transduction of rapid (half times of 10-30 min) irradiance-dependent changes of *CAO* and *Lhcb* mRNA abundance.

A slower response and a longer-term regulation of *CAO* and *Lhcb* gene expression may be exerted by the redox-state of the plastoquinone pool (Allen et al., 1995; Melis 1998). This signaling pathway accounted for changes in cellular Chl and mRNA levels that were less pronounced than those from the rapid induction described earlier in this work. Thus, a rapid canonical signaling pathway and a longer-term signal transduction derived from the redox state of the PQ pool may function cooperatively or independently in the photo-acclimation process. The prompt response may be important for changes in the Chl antenna size within a few hours to confer acclimation to sudden and drastic environmental irradiance changes. The longer-term acclimation response may be important for total coordination of chloroplast functions, such as optimization of photosystem composition, phosphorylation of LHC-II and PSII-core proteins, and transcription of chloroplast-encoded genes (Allen et al., 1995; Melis, 1998).

Since the two different signaling pathways ultimately affect the transcription of the *CAO* and *Lhcb* genes, it would be of interest to analyze the 5'-promoter region of these genes in search of binding domains for irradiance-dependent regulatory factors. It was reported that a G-like box is present in the *Lhcb* genes of *D. tertiolecta* and is involved in the binding of light-intensity-dependent regulatory factor(s) (Escoubas et al., 1995). It was postulated that phosphorylation by a protein kinase enables the binding of this unknown trans-factor to the G-like box, thus repressing the *Lhcb* transcription in response to the redox state of the PG pool. A comparison of the 5' -region sequences of the *CAO* gene in *D. salina* and *C. reinhardtii* with those of *Lhcb* (*cab*) genes from *D. tertiolecta* (database searches of plant cis-acting regulatory DNA elements (PLACE; Higo et al., 1999)) did not reveal any significantly conserved light-responsive elements among these sequences. However, we found two G-like boxes in the 5' -region sequences of *CAO* from *D. salina* and one G-like box in *C. reinhardtii* (data not shown). The G-like boxes had the consensus sequence " ACGTG" . It is possible that a putative transcriptional repressor binds to this G-like box as a link in the signal transduction pathway, which is initiated by the redox state of the plastoquinone pool (long-term photoacclimation). In the short-term photoacclimation (Fig. 10), activation of CDPK is involved in the transcriptional activation of *CAO* and *Lhcb*. In this case, it is possible that a different trans-activator competitively binds to this motif and/or modulates the binding of the repressor in order to activate the transcription of *CAO* and *Lhcb*. Obviously, more work is needed to fully dissect the function of the two signal transduction mechanisms. We should note that such a G-like box sequence was not found in the 5'-promoter region of *GSA* gene in *C. reinhardtii* (Matters and Beale, 1994), indicating that different trans-factor(s) might be responsible for the blue-light induction of the *GSA* gene transcription.

In summary, work in this manuscript delineated the operation of two distinct signal transduction pathways for the regulation of *CAO* and *Lhcb* gene expression in response to irradiance. These mechanisms function, respectively, in the short-term and long-term acclimation of chloroplasts and play a role in the adjustment of the Chl antenna size in *D. salina* in response to irradiance.

A cytosolic signal transduction pathway appears to be responsible for short-term acclimation responses, whereas a chloroplastic signal transduction pathway is responsible for longer-term acclimation responses. Continued dissection and analysis of these mechanisms and their coordination is expected to provide additional useful information about the signal transduction pathway and about the molecular mechanism for the regulation of the Chl antenna size in green algae. Finally, pharmacological effector molecules could prove to be useful in the characterization of mutants with altered responses to light intensity, resulting upon modification and/or interference with the mechanism of irradiance perception and signal transduction.

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