Light intensity regulation of *cab* gene transcription is signaled by the redox state of the plastoquinone pool

(algae/phosphorylation/photoacclimation)

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ABSTRACT   The eukaryotic green alga *Dunaliella terti-olecta* acclimates to decreased growth irradiance by increasing cellular levels of light-harvesting chlorophyll protein complex apoproteins associated with photosystem II (LHCIIs), whereas increased growth irradiance elicits the opposite response. Nuclear run-on transcription assays and measurements of *cab* mRNA stability established that light intensity-dependent changes in LHCII are controlled at the level of transcription. *cab* gene transcription in high-intensity light was partially enhanced by reducing plastoquinone with 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), whereas it was repressed in low-intensity light by partially inhibiting the oxidation of plastoquinol with 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB). Uncouplers of photosynthetic electron transport and inhibition of water splitting had no effect on LHCII levels. These results strongly implicate the redox state of the plastoquinone pool in the chloroplast as a photon-sensing system that is coupled to the light-intensity regulation of nuclear-encoded *cab* gene transcription. The accumulation of cellular chlorophyll at low-intensity light can be blocked with cytoplasmically directed phosphatase inhibitors, such as okadaic acid, microcystin L-R, and tautomycin. Gel mobility-shift assays revealed that cells grown in high-intensity light contained proteins that bind to the promoter region of a *cab* gene carrying sequences homologous to higher plant light-responsive elements. On the basis of these experimental results, we propose a model for a light intensity signaling system where *cab* gene expression is reversibly repressed by a phosphorylated factor coupled to the redox status of plastoquinone through a chloroplast protein kinase.

Photosynthetic organisms physiologically compensate for changes in growth irradiance by altering the abundance of specific components in the photosynthetic apparatus (1–3). This phenomenon of photoacclimation requires the organism to sense differences in light intensity, convert that signal to biochemical information, and transfer the information to the regulatory elements responsible for gene expression. Photoacclimation specifically leads to changes in the abundance of light-harvesting chlorophyll protein complexes (LHCs) and distinctly differs from light-induced developmental processes— e.g., greening of chloroplasts—in that the changes are repeatedly reversible (4) and occur in fully differentiated cells (5–7). In the unicellular chlorophyte alga *Dunaliella tertiolecta* photoacclimation is readily monitored by measuring changes in chlorophyll a (Chl-a) per cell. Indeed, transferring a culture grown under high-intensity light (HL; 700 μmol of quanta·m^{-2}·s^{-1}) to low-intensity light (LL; 70 μmol of quanta·m^{-2}·s^{-1}) induces a 3-fold increase in cellular chlorophyll (Chl) within 24 h (4, 8). The increases in pigment are related to parallel changes in the abundance of LHC apoprotein associated with photosystem II (PSII) (LHCII) (9). A 3- to 4-fold increase in *cab* mRNA occurs

within 9 h and precedes the accumulation of the apoproteins (10). The changes in mRNA were hypothesized to be a consequence of repression of nuclear *cab* gene expression at high-growth irradiance levels (10). Here, we report that *cab* gene transcription is coupled to irradiance through the redox status of the plasto-quinone pool.¶

MATERIALS AND METHODS

Restriction endonucleases and the Random Primers DNA labeling system were obtained from Bethesda Research Laboratories. [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) and [α-^{32}P]UTP (800 Ci/mmol) were purchased from DuPont/New England Nuclear. DNA sequencing was performed by using Sequenase version 2.0 DNA sequencing kit from United States Biochemical. Plasmid DNA used for DNA sequencing was purified with the Plasmid Midi kit from Qiagen (Chatsworth, CA).

Culture Conditions. *D. tertiolecta* (Woods Hole clone DUN) was grown in continuous cultures at 18°C in 3.2-liter turbidostats as described (11–13) and kept optically thin (3–5 × 10^5 cells per ml). For kinetic studies, growth irradiance was shifted in a single step from 700 (HL) to 70 (LL) μmol of quanta·m^{-2}·s^{-1} (4). Pigments were measured in 90% acetone extracts as described (14).

Inhibitor Studies. The effects of photosynthetic electron-transport inhibitors 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP), methylamine, and hydroxylamine were examined over a range from 10^{-8} to 10^{-4} M. For each inhibitor, quintuplicate experiments were performed, and cell growth rate and total cellular Chl concentration were followed. Hydrophobic inhibitors were dissolved in dimethyl sulfoxide (DMSO) and delivered to give a final DMSO concentration of 100 μM. Identical levels of DMSO were added to controls and had no effect on cell growth or Chl pools (15). The effects of phosphatase inhibitors were examined as described above.

Measurement of *cab* mRNA Stability. Cells acclimated to HL or LL were incubated with actinomycin D dissolved in 1.5 mM DMSO/0.28% ethanol (final actinomycin D concentration, 75 μg/ml). At given time intervals following the addition of actinomycin D, total RNA was extracted as described (16).

Total RNA was dot blotted on positively charged nylon membranes or fractionated by electrophoresis through 1.1% agarose/formaldehyde gels and transferred to the membrane (17). The *cab*-specific DNA probe [1-kb *Eco*RI DNA restriction fragment containing the *cab1* cDNA from *D. tertiolecta* (18)] and a ribosomal-specific DNA probe (3.6-kb *Bam*HI restriction fragment from the plasmid p-92B containing the ribosomal genes from *Chlamydomonas reinhardtii* were radiolabeled, the blots were hybridized, and the signals were quantified with a PhosphorImager scanner (Molecular Dynamics).

Nuclear Run-on Transcription Assays. Intact nuclei were isolated as described (19), except that the Percoll gradient was omitted. The supernatant from the cell lysis was reserved for cytoplasmic RNA extraction, as described above. The pellet containing intact nuclei was resuspended in 2.5% (wt/vol) Ficoll/0.5 M sorbitol/0.008% spermidine/5 mM MgCl$_2$/10 mM Tris·HCl, pH 7.5/50% (vol/vol) glycerol/1 mM dithiothreitol, frozen in liquid N$_2$, and stored at $-80°C$. The yields were in the range of 25% as determined by counting 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei with an epifluorescence microscope.

In vitro RNA transcription from 1×10^8 nuclei was measured as described (19). Incubation time was 90 min at 30°C. Radioactively labeled RNA was extracted as described (16), and analysis by formaldehyde/agarose gel electrophoresis indicated that the *in vitro* labeled transcripts represented a continuous spectrum of sizes from 0.1 to 3 kb. The omission of unlabeled nucleotides (ATP, GTP, and CTP) or the addition of actinomycin D (20 μg/ml) resulted in decreased incorporation of [α-^{32}P]UTP into RNA by 90% and 80%, respectively, indicating that the incorporation of radioactivity was due to *de novo* RNA synthesis.

The ^{32}P-labeled transcripts were used as probes on filters containing an excess of selected target DNA. Filters were prepared by dot blotting 10 μg of linearized, denatured plasmid DNA containing either the *cab1* gene of *D. tertiolecta* or the 25S, 5.8S, and 18S rRNA genes of *C. reinhardtii*. Hybridization was carried out at 42°C for 48 h with $1-2 \times 10^7$ cpm of labeled RNA probe. Filters were washed for 15 min at room temperature in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS, for 15 min at room temperature in 0.1× SSC/0.1% SDS, for 30 min at 37°C in 0.1× SSC/10 μg of RNase A per ml, and for 60 min at 60°C in 0.1× SSC/0.1% SDS. Air dried filters were exposed to x-ray film at $-80°C$ with intensifying screens. Radiolabeling was quantified with a PhosphorImager.

Gel-Shift Assays. Cells from 500-ml cultures were harvested by centrifugation and resuspended in 0.5 ml of lysis buffer at 0–4°C containing 25 mM Hepes·KOH (pH 7.9), 0.6 M KCl, 12 mM 2-mercaptoethanol, 0.12 mM EDTA, 0.24 M sucrose, 0.84 mM MgOAc, 2 mM phenylmethylsulfonyl fluoride, and 1 μg each of antipain and leupeptin per ml. Cells were disrupted at 5°C with 0.3 ml of 0.5-mm glass beads in a Biospec Products (Bartlesville, OK) cell disrupter, and the extract was clarified by ultracentrifugation at 100,000 × *g* for 1 h at 4°C. The supernatant was fractionated with (NH$_4$)$_2$SO$_4$ at 0–30% saturation (F30), 30–60% saturation (F60), and >60% saturation (Fsup). Following centrifugation, the fractionated proteins were resuspended in 0.5 ml of storage buffer [25 mM Hepes·KOH, pH 7.9/40 mM KCl/5 mM 2-mercaptoethanol/ 0.1 mM EDTA/10% (vol/vol) glycerol] and dialyzed against the same buffer to remove (NH$_4$)$_2$SO$_4$. Protein was measured by using the bicinchoninic acid method (20).

DNA probes were labeled with ^{32}P by PCR using pBluecab1, a plasmid carrying the upstream region of the *cab1* gene, as a template as described (21). Four oligonucleotides, designated A (5'-GCTCTAAAGTTGAAG-3'), B (5'-GCTGACAT-TCTAACG-3'), C (5'-CCCTTCCGAGTGTCTG-3') and D (5'-CATTGGCTCTCACATCAG-3'), were used as PCR primers. DNA probes were purified by electrophoresis through

2.5% agarose gels, and the incorporation of ^{32}P was measured by liquid scintillation counting.

For binding studies, 20 μl of PCR-amplified DNA (10^4 cpm), 3 μg of poly(dI-dC)·poly(dI-dC), 45 mM KCl, 25 mM Hepes (pH 7.9), 1.1 mM EDTA, 0.5 mM dithiothreitol, and 5% (vol/vol) glycerol were combined with 10 μg of protein extract and incubated at room temperature for 30 min. The reaction mixtures were loaded on 4% polyacrylamide gels and electrophoresis was carried out for 2.5 h at a constant voltage of 10 V/cm. Gels were fixed for 30 min in 20% (vol/vol) methanol/ 10% (vol/vol) acetic acid, dried, and autoradiographed (22).

RESULTS

RNA Stability. Following a shift from HL to LL, *cab* mRNA levels typically increased 3- to 4-fold within 9 h (10). The relative stability of mRNA with time was assessed in both HL- and LL-adapted cells that had been pretreated with actinomycin D for 1 h. The half-lives of the *cab* transcripts were not statistically significantly different for the two light conditions.

Effects of Light Intensity on *cab* mRNA Transcription. The rate of *cab* transcription was estimated from run-on transcription assays (19) using nuclei isolated from cells grown in HL or grown in HL and shifted to LL for 3, 6, or 9 h. The results show that following a shift to LL, *cab* gene transcription increased 3-fold, reaching a maximum after 6 h in LL (Fig. 1). Concomitant measurements of *cab* mRNA revealed a 3-fold increase within the first 9 h following the HL to LL shift. Taken together, the results of the RNA stability measurements and the run-on transcription assays establish that the light-intensity-dependent changes in *cab* mRNA levels are primarily a consequence of change in the rate of transcription.

Effects of Photosynthetic Electron-Transport Inhibitors on Cellular Chl and *cab* Gene Expression. Sublethal concentrations of DCMU mimic the effects of acclimation to LL in both eukaryotic (23) and prokaryotic (24) algae. Here, we investigated the effects of five photosynthetic electron-transport inhibitors, including DCMU, on photoacclimation in *D. tertiolecta* (Fig. 2*A*). When cells were grown in HL, partial inhibition of plastoquinone reduction with DCMU induced a 2-fold increase in cellular Chl within 12 h, while sublethal concentrations of the plastoquinone analog DBMIB had no effect on pigment levels (Fig. 2*B*). When 10^{-7} M DBMIB was added to cells grown in LL, cellular Chl levels decreased by
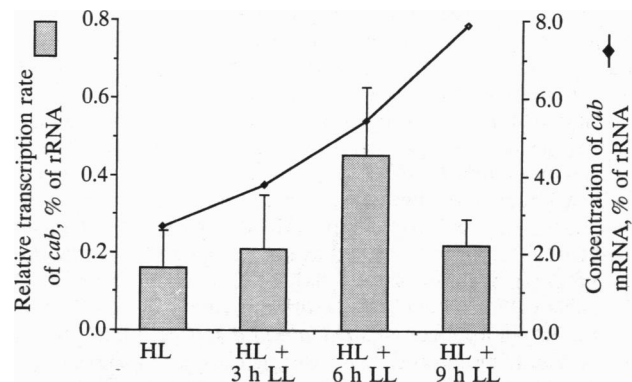

FIG. 1. Measurement of *cab* gene transcription in nuclei isolated from *D. tertiolecta* during photoacclimation. Cells were grown in HL (700 μmol of quanta·m^{-2}·s^{-1}), and the cultures were either harvested or shifted to LL (70 μmol of quanta·m^{-2}·s^{-1}) for 3, 6, or 9 h. The three plasmids used as probes were as follows: p-92B, containing rDNA genes of *C. reinhardtii*; pDTcab1, containing the *cab1* gene; and pT$_7$/T$_3$ as a negative control. The *cab* transcripts were normalized to rRNA. For each time point, total RNA was purified, and *cab* transcript concentration was determined by dot-blot hybridization and normalized to the concentration of rRNA transcripts. The data are presented as the mean ± 1 SD (*n* = 3).
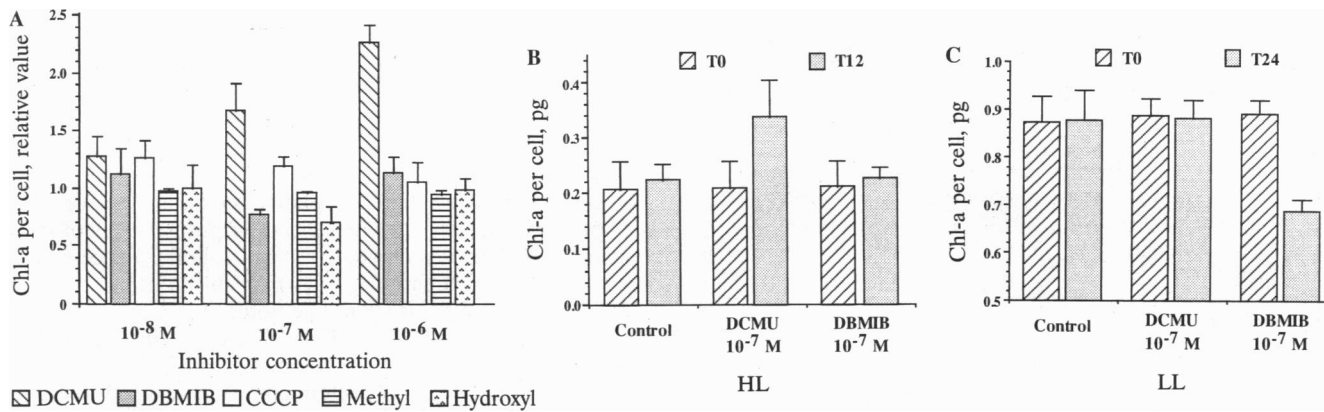
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FIG. 2. Effect of electron-transport inhibitors on the level of Chl-a per cell. (*A*) Cells were grown in HL (700 μmol of quanta·m^{-2}·s^{-1}). The culture was split, and inhibitors were added to the final concentrations indicated. The amount of Chl-a per cell and cell density (data not shown) were measured immediately after the addition of inhibitor and 48 h later. Data are presented as the mean ± 1 SD (n = 2–5). Methyl, methylamine; Hydroxyl, hydroxylamine. (*B*) Comparison of the effect of DCMU or DBMIB on Chl-a in HL-grown cells. The experimental procedure is the same as in *A*, except that the Chl-a measurements were made 0 h (T0) and 12 h (T12) after inhibitor addition. Data are presented as the mean ± 1 SD (n = 8). (*C*) Comparison of the effect of DCMU or DBMIB on Chl-a in LL-grown cells (LL = 70 μmol of quanta·m^{-2}·s^{-1}). Measurements were made 0 h (T0) and 24 h (T24) after inhibitor addition. Data are presented as the mean ± 1 SD (n = 8).

25%, whereas DCMU had no effect (Fig. 2*C*). The uncouplers of photophosphorylation CCCP and methylamine and hydroxylamine, an inhibitor of water oxidation (see Fig. 5 for the action sites of inhibitors), did not alter cellular Chl levels. All inhibitors led to reductions in growth rates that were nonlinearly related to concentration (data not shown), thereby confirming their permeation and efficacy. The lack of effect with CCCP, methylamine, or hydroxylamine suggests that neither ATP synthesis nor water splitting *per se* are directly involved in signaling *cab* gene expression.

While DCMU specifically inhibits the oxidation of the primary electron acceptor of PSII, Q_A (and consequently the reduction of the secondary acceptor, plastoquinone), DBMIB can, in principle, inhibit both photosynthetic and mitochondrial electron transport (25). In the photosynthetic electron-transport chain, DBMIB reduces the rate of oxidation of plastoquinol by competitively binding to the cytochrome b_6/f complex (26, 27). Using oxygen evolution and consumption in the light and dark to assess the degree of inhibition of these two electron pathways, we found that at 10^{-7} M the inhibition of photosynthetic electron transport was greater than 50%, while an effect on mitochondrial electron transport was undetectable (results not shown).

In cells grown at a constant light intensity, DCMU induced a doubling of the *cab* mRNA abundance, mimicking the effect of a shift to a lower light intensity, whereas DBMIB induced a 75% decrease in *cab* mRNA abundance, mimicking the effect of a shift to a higher irradiance (Fig. 3).

The Potential Role of Protein Phosphorylation in the Signal-Transduction Pathway. The redox state of the plastoquinone pool controls a thylakoid protein kinase that reversibly phosphorylates LHCII (28), as well as other thylakoid and stromal proteins (29, 30). We hypothesized that if repression of *cab* genes was mediated by a redox-controlled protein kinase, then the derepression—i.e., enhancement—of *cab* gene expression at LL could be affected by inhibitors of protein phosphatases. The addition of 1–100 nM okadaic acid, microcystin-LR, or tautomycin inhibited the increase of Chl-a per cell by 33–56% within 24 h. Growth rate was not significantly affected over this period (data not shown). Okadaic acid and microcystin-LR primarily inhibit a protein phosphatase designated type 2A (PP2A) (31), while tautomycin primarily represses type 1 phosphatase (32, 33). Neither type 1 nor type 2A protein phosphatase activity has been detected in chloroplasts (34, 35). Thus, our results suggest that both type 1 and type 2A phosphatases are involved in the signal-transduction

pathway and that the phosphatases are located in the cytoplasm and/or nucleus.

Promoter Elements and DNA-Binding Proteins. Sequence analysis of the promoter region of two of the *cab* genes reveals motifs homologous with the AT-1, G, and GT boxes of higher plants (Fig. 4*A*). We used a gel mobility-shift assay to investigate if the motifs homologous to these light-responsive elements of higher plants corresponded to DNA-binding sites for factors involved in light-intensity regulation of *cab* genes.

Two overlapping DNA fragments containing elements homologous to either the AT-1 and G boxes (Fragment I) or G and GT boxes were used for gel-shift assays (Fig. 4*B*). Identical gel mobility-retarded bands were observed for the F60 and Fsup protein fractions from HL and LL cells (data not shown), whereas the mobility patterns for F30 fractions were different. DNA–protein complexes for both fragments were detected only in the F30 HL extracts (Fig. 4*C*). As the two fragments
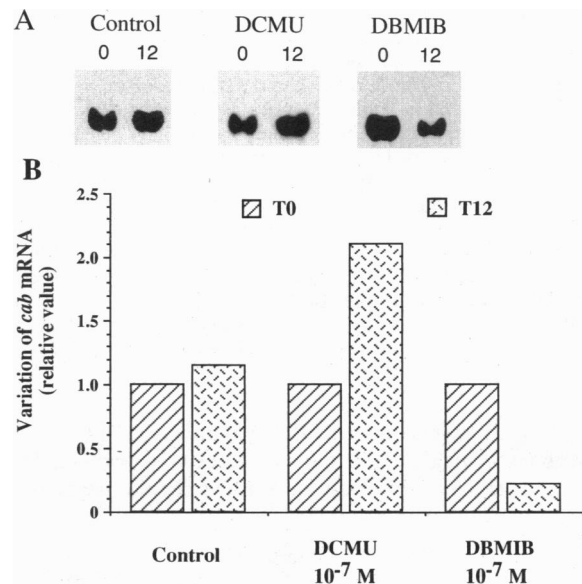

FIG. 3. Effect of electron-transport inhibitors on *cab* mRNA abundance. Conditions were as described in the legend to Fig. 2. DCMU or DBMIB was added to a final concentration of 10^{-7} M, and the cells were grown for 12 h in HL. (*A*) Autoradiograph of Northern blots. (*B*) Histogram of the quantitative results from two DCMU and three DBMIB experiments.
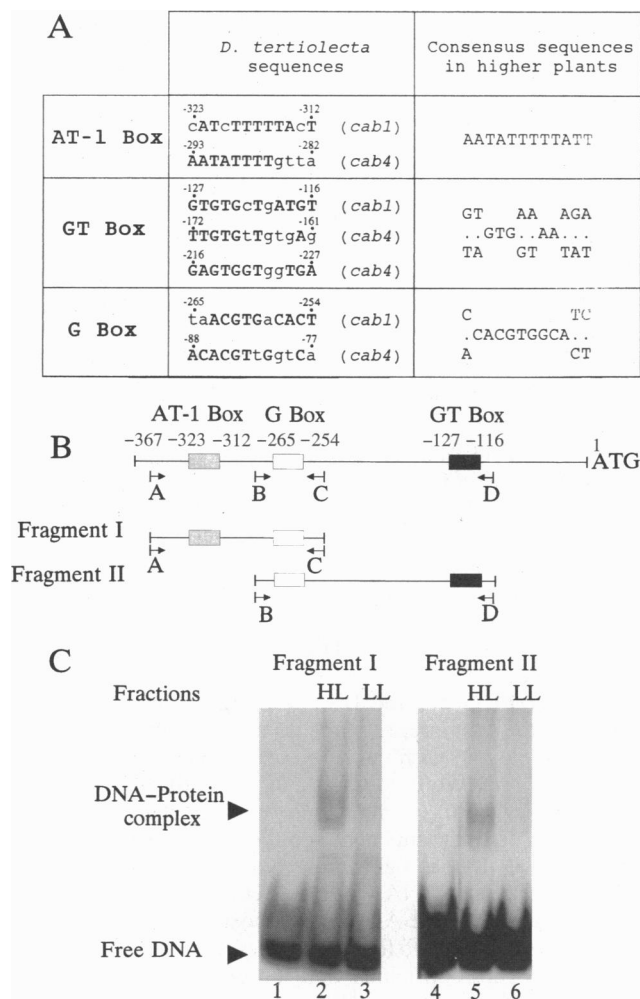
FIG. 4. (*A*) Sequence similarity between promoter of *D. tertiolecta* *cab* gene and promoter of light regulated genes from higher plants. Sequence comparison of AT-1 (36, 37), G (38, 39), and GT boxes (38–42) from higher plants showing similarity with the motifs found in the promoter region of *D. tertiolecta* *cab1* and *cab4* genes (two of the *cab* genes in this species; GenBank accession numbers U28718 and U28725, respectively). The numbers above the initial and final bases are the nucleotide positions relative to the start codon of the respective gene. Bases denoted in boldface, uppercase letters are identical with the consensus sequence in higher plants. (*B*) Diagram showing the positions of fragments I (137 bp) and II (168 bp) relative to the promoter region and putative light-regulated elements of *cab1*. The oligonucleotide primers designated A–D (described in *Materials and Methods*) were used to construct the fragments. (*C*) Gel mobility-shift assay performed with F30 protein fraction isolated from HL (lanes 2 and 5) or LL (lanes 3 and 6) showing DNA–protein complexes specific for the HL protein extract only. Both DNA fragments I (lanes 1, 2, and 3) and II (lanes 4, 5, and 6) gave similar results. Lanes 1 and 4 represent the DNA fragments I and II, respectively, in the absence of protein extract.

overlap by 31 bases, of which 12 bases consist of a G-like box, these results suggest that the G-like box is involved in the binding of the light-intensity-dependent regulatory factor(s).

DISCUSSION

The results presented here demonstrate the following: (*i*) the light-intensity-dependent changes in LHCII are due to changes in *cab* gene transcription; (*ii*) the effect of irradiance can be pharmacologically simulated by reversible manipulation of the redox status of the plastoquinone pool in the chloroplast; (*iii*) inhibition of protein phosphatases reduces the increase in Chl

observed after a shift from HL to LL; (*iv*) a 250-bp region (−166 to −364) from the *cab1* promoter contains three motifs homologous to light-responsive elements of higher plants; and (*v*) a region containing a G-like box binds one or more protein factors present in HL- but not LL-grown cells. We discuss these results in the context of the signal-transduction pathway for photoacclimation.

During greening of higher plants, the abundance of LHCII is primarily a consequence of transcriptional control of *cab* genes (43). Our results confirm that a similar process occurs during photoacclimation to LL in a fully differentiated eukaryotic green alga. It should be noted that photoacclimation from LL to HL and from HL to LL are not inverse processes on a physiological level (44). In an HL to LL shift, the rate of synthesis of the pigment protein complexes exceeds the decelerating division rate (4, 11). Changes in *cab* transcription during an LL to HL transition, however, are not brought about by an acceleration of division rate but rather by repression of *cab* gene expression and increased degradation of the message. DCMU and DBMIB block the reduction and oxidation, respectively, of the plastoquinone pool, and no other site of inhibition tested had an effect on cellular Chl levels. These results strongly imply that the redox status of the plastoquinone pool is the initial signal in the transduction process.

On the basis of the molecular and pharmacological results presented here, we propose a working model for photoacclimation in eukaryotic cells (Fig. 5). The oxidation of plastoquinone is the slowest step in photosynthetic electron-transport chain between PSII and PSI (45, 46). When irradiance is increased, the rate of photochemistry by PSII exceeds
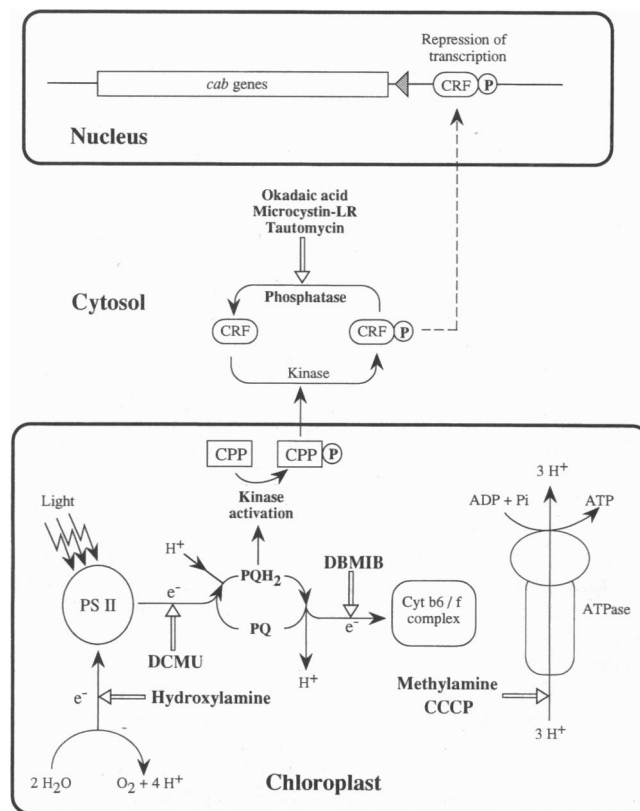

FIG. 5. Model for interorganellar network allowing the redox regulation of nuclear gene transcription by a chloroplastic photoreceptor (see text). CPP, chloroplastic phosphoprotein; CRF, *cab* gene repressor factor. The sites of action of the photosynthetic electron-transport inhibitors (hydroxylamine, DCMU, DBMIB, methylamine, and CCCP), as well as the proposed action sites for the protein phosphatase inhibitors (okadaic acid, microcystin-LR, and tautomycin), are indicated.

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the rate of oxidation of the plastoquinol (47). In effect, the ratio of plastoquinone to plastoquinol is a biological light meter that senses the ratio of the rate of light absorption by the photosynthetic apparatus to the rate of linear photosynthetic electron transport.

We hypothesize that the signal of the redox state of plastoquinone is communicated from the plastid to the nucleus via a phosphorylation cascade. In our model, the redox-sensing kinase phosphorylates a soluble chloroplastic phosphoprotein (CPP) (Fig. 5). The unphosphorylated form of CPP is associated with the thylakoid membrane, while its phosphorylated form is released to the stroma (48). The phosphorylated form of CPP may diffuse across the chloroplastic membrane or may act through a secondary messenger to activate a cytoplasmic protein kinase. We hypothesize that the cytoplasmic protein kinase phosphorylates a *cab* gene repressor factor (CRF) which, in its phosphorylated form, is translocated in the nucleus, where it binds to the *cab* gene promoter region and represses transcription. This hypothesis is supported by the gel-shift experiments showing that light-irradiance levels control the binding activity of a factor on the *cab1* promoter region. This part of the promoter region contains a G-like box. In the higher plants, the G-box-binding factor activity is controlled by light via phosphorylation (38). Following a shift to LL, the plastoquinone pool becomes largely oxidized, and the activity of the linked protein kinase is low. Consequently, the rate of phosphorylation of CPP is expected to be low in this model. The derepression of *cab* gene expression in LL would require the dephosphorylation of CRF in the cytoplasm or nucleus. Indeed, inhibition of protein phosphatase activity resulted in diminished photoacclimation response to LL, supporting this hypothesis.

Our model specifically explains how light intensity is perceived by the photosynthetic apparatus and couples photosynthetic electron transport to modification of nuclear gene expression (49). On short time scales (minutes), when the rate of photon absorption by PSII exceeds the rate of oxidation of plastoquinol, a kinase, coupled to the redox status of the plastoquinone pool, phosphorylates LHCII, leading to a reduction in the effective photon absorption cross-section for PSII (29, 30, 50). The conferred changes in cross-section are ≈10–15% and lead to proportional changes in the rate of excitation delivery to PSII. On longer time scales, the same signal-transduction pathway can induce transcriptional control of the genes encoding LHCII. Thus, the plastoquinone pool exerts both short-term, fine control on the light-harvesting system via posttranslational protein phosphorylation and long-term, coarse control over LHCII protein abundance via transcriptional regulation. This model predicts that any environmental parameter altering the balance between plastoquinone reduction and plastoquinol oxidation will affect *cab* gene expression. For example, cells grown at low or high temperature at a constant irradiance would exhibit similar differences in Chl content as HL- and LL-adapted cells, respectively (51, 52).

The model presented here is a working hypothesis. While it accommodates the basic features of the experimental results, proof that the signal from the plastoquinone redox poise is transmitted via a phosphorylation cascade and the characterization of the regulatory elements require much more experimental data. Nonetheless, our results provide the evidence that the redox state of a specific electron-transport component in the plastid modifies the expression of a nuclear-encoded gene. This signaling pathway appears to be part of a larger system of redox control of gene expression in photosynthetic organisms (53, 54).

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