Plastid Regulation of *Lhcb1* Transcription in the Chlorophyte Alga *Dunaliella tertiolecta*[1]

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We identify four novel DNA-binding complexes in the nuclear-encoded *Lhcb1* promoter of the chlorophyte alga *Dunaliella tertiolecta* that are regulated by photosynthetic pathways in the plastid. The binding activities of three of the complexes were positively correlated with time-dependent changes in *Lhcb1* transcript abundance, implicating their roles as transcriptional enhancers in a retrograde signal transduction pathway. Using a combination of inhibitors, uncouplers, and antimycin A, and by following the kinetic pattern of gene regulation, we infer two different sensors in the signal transduction pathway. On short time scales of 0.5 to about 4 h, the transthylakoid membrane potential appears to be a critical determinant of gene expression, whereas on time scales of 8 h or longer, the redox state of the plastoquinone pool becomes increasingly more important. The differentiation of these two types of signals was observed in parallel effects on gene transcription and on the patterns of DNA-binding activities in the *Lhcb1* promoter. These signals appear to be transduced at the nuclear level via a coordinated ensemble of DNA-binding complexes located between -367 and -188 bp from the start codon of the gene. The regulation of these elements allows the cell to up- or down-regulate the expression on *Lhcb1* in response to changes in irradiance.

All photosynthetic organisms can adjust the rate of photon absorption to optimize photosynthetic electron transport (PET) in relation to changes in spectral irradiance. Long and persistent shifts in photon flux density (PFD) often involve changes in gene expression that modify the activity, concentration, and structure of the photosynthetic machinery (Falkowski and LaRoche, 1991; Anderson et al., 1995; Durnford and Falkowski, 1997). In comparison with higher plants, photoacclimation is far more pronounced in unicellular algae, where not only the magnitude and rates of the responses are extraordinary, but also the response can easily be distinguished from developmental processes (Falkowski and Chen, 2003). In particular, species in the marine unicellular chlorophyte genus *Dunaliella* can undergo an almost 10-fold change in the abundance of antenna chlorophyll (Chl)-binding proteins on a per cell basis within a single generation (Sukenik et al., 1988). In eukaryotic algae, these changes involve transcription and translation of both nuclear and chloroplast genes. Consequently, tight interorganellar regulatory networks are required to coordinate gene expression in these two compartments. In this paper, we examine how changes in the abundance of the nuclear-encoded *Lhcb* gene in response to changes in PFD are sensed by the plastid and how the signals are correlated with binding factors in the promoter region.

At least three signaling pathways involved in plastid regulation of nuclear gene expression have been described for plant cells. The first is mediated by tetrapyrrole biosynthesis, the second requires plastid protein synthesis, and the third involves electron carrier redox poise and potentially other photosynthetic signals (for review, see Rodermel, 2001; Surpin et al., 2002; Gray et al., 2003). The first two pathways play important roles in developmental processes and in response to dark/light cycles, whereas chloroplast redox signaling appears to control the nuclear photosynthetic gene expression during the acclimation to changes in PFD, temperature, and CO_2 (Durnford and Falkowski, 1997; Huner et al., 1998; Surpin et al., 2002; Strand et al., 2003).

Among the thylakoid electron carriers, the plastoquinone (PQ) pool is considered to be an ideal candidate for signaling excess or insufficient PSII activity relative to the capacity for carbon fixation (Falkowski et al., 1986; Pfannschmidt et al., 1999). Because there are several PQ molecules per reaction center, and the oxidation of PQH_2 is the slowest reaction within the PET chain, the redox state of the PQ pool is sensitive to both excitation pressure on the donor side of PSII and downstream sink capacity on the acceptor side of PSI. Hence, the ratio of PQ/PQH_2 is a biological light meter that reflects the ratio between the rate of light absorption by the photosynthetic apparatus and the rate of linear photosynthetic electron transfer (Falkowski et al., 1986; Fujita et al., 1987; Escoubas et al., 1995).

Using a variety of PET and other inhibitors, Escoubas et al. (1995) demonstrated that *Lhcb1* expression is dependent on the redox state of the PQ pool in *Dunaliella tertiolecta*. Subsequently, redox regulation of *Lhcb1* and other nuclear-encoded genes were reported in algae and higher plants (Maxwell et al., 1995; Karpinski et al., 1997, 1999; Pfannschmidt et al., 2001). However, it is unclear whether the PQ pool is the only chloroplastic signal initiator and how the signal is transduced. It has been suggested that the site of control involves a stromal-redox component, such as ferredoxin, thioredoxin, and/or glutathione (Danon and Mayfield, 1994; Montané et al., 1998; Oswald et al., 2001). The role of the cytochrome $b_6 f$ complex-mediated light-harvesting complex (LHC)II kinase as a possible redox sensor independent of the PQ pool has also been proposed (Kovács et al., 2000; Rintamäki et al., 2000; Pursiheimo et al., 2001; Yang et al., 2001; Zer and Ohad, 2003). In addition to controlling the xanthophyll cycle-mediated nonphotochemical quenching (Demmig-Adams et al., 1996), the transthylakoid membrane pH gradient (ΔpH) has been shown to control the phosphorylation of LHCII (Fernyhough et al., 1984) and regulates nuclear-encoded photosynthetic genes (Horton et al., 1996; Wilson and Huner, 2000). Furthermore, chloroplast ATP levels have been suggested to play roles in the signal transduction pathway that regulates LHCII kinase/phosphatase activities and state transitions (Baker et al., 1982; Fernyhough et al., 1984; Bulté et al., 1990; Wollman, 2001), as well as in adjusting the ratio between linear and cyclic electron transfer (Joliot and Joliot, 2002).

The mechanism(s) by which a chloroplast redox signal is relayed to the nucleus remains unknown. Plastid redox signals may be transmitted via one or more plastid factors, the existence of which was postulated decades ago (Bradbeer et al., 1979). The involvement of one or more phosphorylatable protein messenger(s) in the chlorophyte algae, *D. tertiolecta* (Escoubas et al., 1995) and *Chlamydomonas reinhardtii* (Depège et al., 2003), as well as the terrestrial plant Arabidopsis (*Arabidopsis thaliana*; Carlberg et al., 2003), has been suggested. It is also possible that redox signaling occurs via the same phosphorelay system, involving heterotrimetric G proteins and protein kinase C, which regulates the transcription of *PsaF* in response to light and the functional state of the plastid (Chandok et al., 2001).

In this study, we measured the kinetics of *Lhcb1* transcript abundance and DNA-binding activity associated with a 180-bp *Lhcb1* promoter region in *D. tertiolecta* following light transitions, or following the addition of a variety of inhibitors, or combinations of the two types of treatments. Our results reveal, for the first time to our knowledge, that the binding activity of multiple DNA-binding complexes, associated with the *Lhcb1* promoter region, are affected by changes in light intensity, redox poise of electron transfer components between PSII and PSI, and the transthylakoid membrane potential. The relationship between the *Lhcb1* transcript abundance and the binding activities of these complexes implicate the roles of the latter as transcriptional enhancers in a retrograde signal transduction pathway. The results further suggest that two types of chloroplastic signals, the redox state of PQ pool as potentially sensed by the cytochrome $b_6 f$ complex and the transthylakoid membrane potential, cooperatively regulate *Lhcb1* expression.

RESULTS

DNA-Binding Patterns in the *Lhcb1* Promoter Region

To dissect the binding properties of the *Lhcb1* promoter, we generated a set of six 30-bp long oligo DNA probes that sequentially represent the 180-bp promoter region (from -367 to -188 bp; Fig. 1). Based on electrophoretic mobility shift assay (EMSA) of whole-cell protein extracts, we identified four different binding complexes that we call HLF, LF1, LF2, and LF3 (Fig. 2). The largest complex, HLF, bound to all six probes, while the second largest complex, LF1, bound to all except Oligo 3. One of the two smaller complexes, LF2, bound strongly to Oligo 3 (-307 to -278 bp) and Oligo 5 (-247 to -218 bp), whereas LF3 bound primarily to Oligo 5. These results were confirmed using more than one dozen PCR-generated probes in the same promoter region (data not shown). Binding of all complexes is specific; they can be outcompeted by their respective unlabeled oligo DNA, but not by nonspecific competitors such as sonicated salmon sperm DNA (Fig. 2).

There are significant sequence similarities between two sections in the targeted promoter region: from -367 to -288 bp and from -270 to -227 bp (Fig. 1). We identified three tentative binding sites in these two nonoverlapping promoter regions. The first, a TCTAA box, has a consensus sequence TCTAAHGT and is located between -365 to -357 bp (within the Oligo 1 probe) and -266 to -258 bp (within the Oligo 4 probe). In both cases, this site is 12 to 15 bp upstream of two other putative binding sites. The second contains a CACT box with a consensus sequence CARRCACTSGRA and overlaps the third, a GGAA box, which has a core sequence ASMMYYGGAA. Among the three putative binding motifs, only the TCTAA box is also found in the *Lhcb1* promoter in *C. reinhardtii* and in *Lhcb2/Lhcb3* promoters in Arabidopsis.

Effects of DCMU on *Lhcb1* Transcript Abundances

The effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on the time course change of *Lhcb1* transcript abundance in cells grown under different light conditions is shown in Figure 3. In both high-light (HL)- and low-light (LL)-acclimated cultures, the addition of 200 nM DCMU (which leads to an approximately 50% reduction in PET through PSII) resulted in a rapid decrease of *Lhcb1* transcript abundance in the first 2 to 4 h. In HL-acclimated cells, the *Lhcb1* transcript levels recovered and increased nearly 2-fold above the pre-
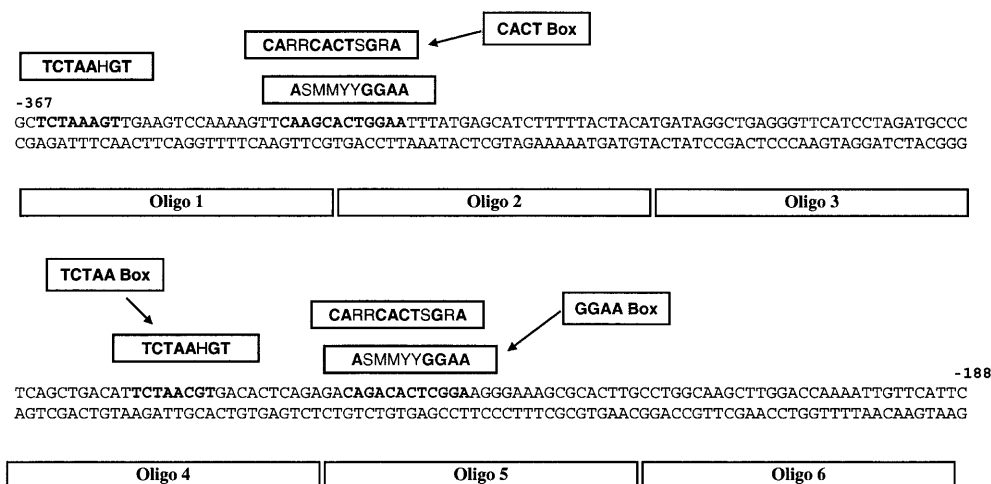
Figure 1. Schematic presentation of the tentative multiple and repetitive binding sites in *D. tertiolecta Lhcb1* 180-bp promoter region (from −367 to −188 bp relative to the starting codon). Six oligo DNA were constructed within the promoter and used as unlabeled competitors or labeled probes in EMSA.

vious steady-state level. The pattern of change in *Lhcb1* transcript abundance is consistent with that of Chl *a*/cell (data not shown). Though the addition of DCMU to HL cultures resulted in increased *Lhcb1* transcript abundance, the pattern differed both in the magnitude and kinetics compared to the control HL-to-LL shift. In the latter, the relative *Lhcb1* transcript levels increased immediately following the light transition, and the level was tripled within the first 4 h, about two times higher than the peak level reached in DCMU-treated cells after 24 h (Fig. 3).

We further tested the ability of DCMU to mimic LL conditions by adding the inhibitor prior to a shift from LL to HL. If the reduction of the PQ pool were the sole signal responsible for the up-regulation of *Lhcb1* transcription, the HL-induced decline in *Lhcb1* transcript abundance should be attenuated. However, DCMU failed to prevent decreases in *Lhcb1* transcript abundance (Fig. 4). These results strongly suggest that the redox state of the PQ pool per se is not the only photosynthetic signal that regulates *Lhcb1* expression.

Effects of Uncouplers on *Lhcb1* Transcript Abundance

The initial decrease in the relative abundance of *Lhcb1* following the addition of DCMU raises the possibility that signals other than the PQ pool redox status are involved in the regulation of *Lhcb1* transcription. In both HL- and LL-acclimated cultures, *Lhcb1* transcript abundance decreased within 1 h following the addition of 400 nM nigericin or 2 μM valinomycin and reached a nadir within 2 h (Fig. 5). Over the next 4 to 6 h, transcript levels rose to approximately 150% of the previous steady-state level.
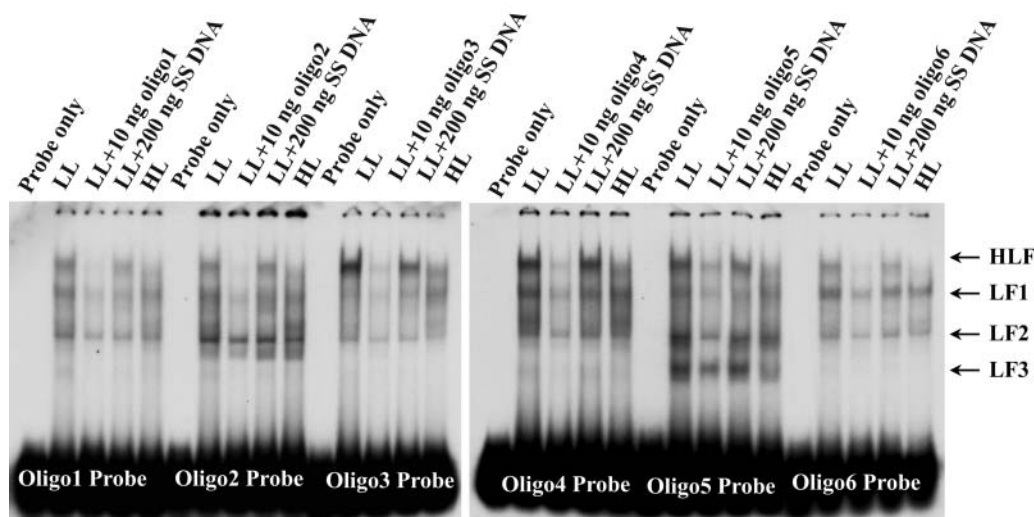

Figure 2. EMSA of DNA-binding activities in the 180-bp *Lhcb1* promoter region (−367 to −188 bp) represented sequentially by Oligo 1 to 6, as well as the competition of the binding activities by specific (unlabeled oligo) and nonspecific DNA fragments. Protein samples were extracted from HL- and LL-acclimated culture and fractionated by a single step with 50% (NH$_4$)$_2$SO$_4$. All binding reactions contained 1 μg of poly(dI.dC).poly(dI.dC). SS DNA, Sonicated salmon sperm DNA fragments.

time, there was a consistently reproducible decline at 2 h (Fig. 6).

Effects of DBMIB on *Lhcb1* Transcript Abundance

In both HL- and LL-acclimated cultures, the addition of 1.5 μM 2,5-dibromo-6-methyl-3-isopropyl-1,4-benzochinon (DBMIB) led to declines in *Lhcb1* transcript abundance (Fig. 7). The transcripts were virtually undetectable 4 h following the addition of the inhibitor and remained so for the rest of incubation. The pattern of the changes in *Lhcb1* transcript abundance was consistent with that of Chl *a*/cell (data not shown).

While the short-term changes of *Lhcb1* transcript abundance are similar between those induced by DBMIB and a shift from LL to HL, the longer-term changes differ. During the acclimation from LL to HL, *Lhcb1* transcript abundance partially recovered after 6 h, reaching a new and lower steady-state level. We further examined the *Lhcb1* changes in HL cells transferred to LL with DBMIB added just before the light shift. Kinetic fluorescence measurements with a fast repetition rate (FRR) fluorometer (Kolber et al., 1998) indicated that DBMIB induced an immediate and dramatic reduction of the PQ pool, followed by a moderate recovery within 2 h. DBMIB effectively prevented *Lhcb1* transcript abundance from increasing due to the reduced irradiance (Fig. 8). These results
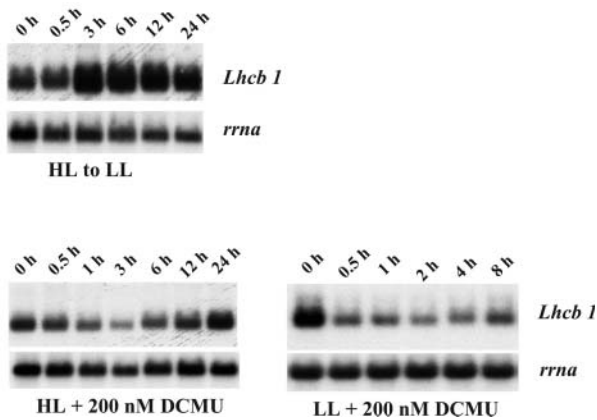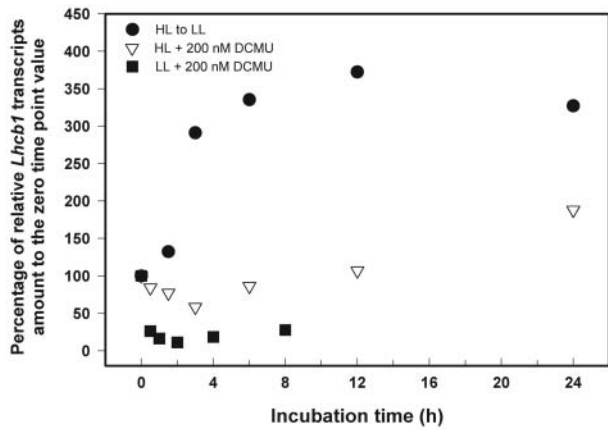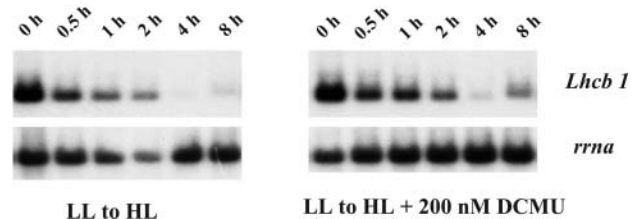
Figure 3. Time course changes in *Lhcb1* and *rrna* transcript levels in HL-acclimated cells following a shift to LL and 200-nM DCMU additions to HL- and LL-acclimated cells. Top, Densitometric analysis of the time course changes in *Lhcb1* level, normalized to *rrna* level, as percentage to the zero time point value. Bottom, Northern-blot analysis of total cellular RNA probed sequentially with *Lhcb1* and *rrna*.

The kinetic patterns of change in *Lhcb1* transcript abundance were similar for the two uncouplers.

Effects of Antimycin A on *Lhcb1* Transcript Abundance

The results from the uncoupler treatments suggest the transthylakoid membrane potential, i.e. the sum of proton gradient and the electric potential, may be involved in the short-term regulation of *Lhcb1* transcription. We further examined this hypothesis by analyzing the effects of antimycin A on *Lhcb1* transcript abundance. Antimycin A inhibits cyclic electron transport around PSI (for review, see Bendall and Manasse, 1995). When 1 μM antimycin A was added to HL cultures, the relative abundance of the *Lhcb1* transcript fluctuated moderately with the overall trend little changed (Fig. 6). However, when 1 μM antimycin A was added to HL cultures together with 200 nM DCMU, *Lhcb1* transcript abundance increased immediately and quadrupled within the next 8 h. Though there was a near linear increase in *Lhcb1* levels over

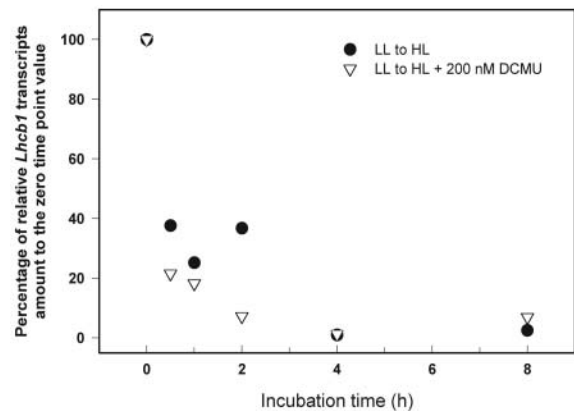**Figure 4.** Time course changes in *Lhcb1* and *rrna* transcript levels in LL-acclimated cells following a shift to HL with or without the presence of 200 nM DCMU. Top, Densitometric analysis of the time course changes in *Lhcb1* level, normalized to *rrna* level, as percentage to the zero time point value. Bottom, Northern-blot analysis of total cellular RNA probed sequentially with *Lhcb1* and *rrna*.

Figure 5. Time course changes in *Lhcb1* and *rrna* transcript levels in LL- and HL-acclimated cells following additions of 400 nM nigericin or 2 μM valinomycin. Top, Densitometric analysis of the time course changes in *Lhcb1* level, normalized to *rrna* level, as percentage to the zero time point value. Bottom, Northern-blot analysis of total cellular RNA probed sequentially with *Lhcb1* and *rrna*.
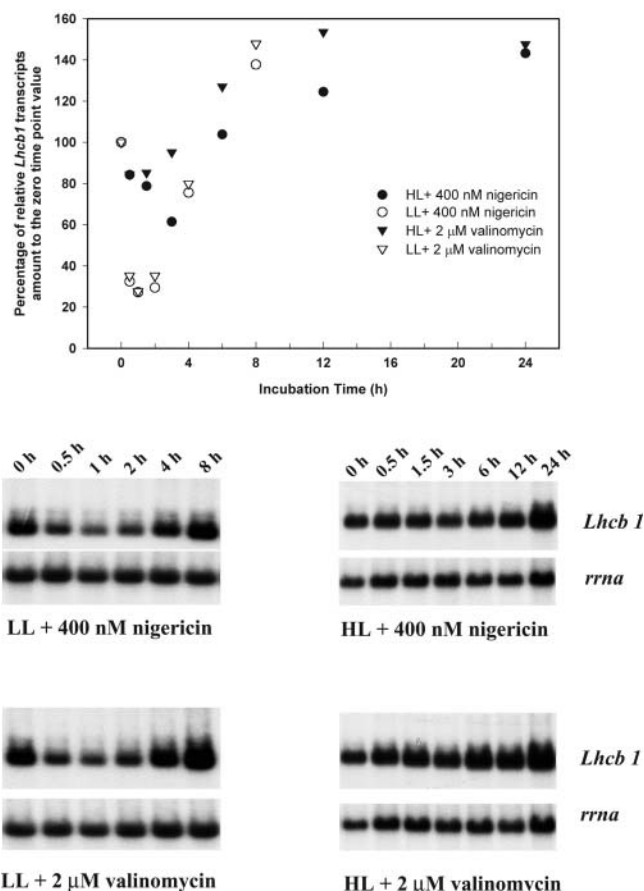
suggest that the signal from the reduced PQ pool repressed the up-regulation of *Lhcb1* transcription that would have otherwise occurred had the shift been done in the absence of the inhibitor.

Effects of Various Treatments on the Redox Status of PQ Pools

Transient Chl fluorescence kinetics was measured to assess the oxidation rate of Q_A^- and the redox status of the PQ pool. The changes in the percentages of oxidized PQ to the PQ pool following the additions of DCMU and DBMIB resembled those following light transitions from HL to LL and from LL to HL respectively (Fig. 9). Addition of DCMU increased oxidized PQ by approximately 50% more than that following the HL-to-LL transition. Addition of DBMIB reduced oxidized PQ by approximately 50% less than that by an LL-to-HL transition. Additions of nigericin, valinomycin, and antimycin A increased oxidation of the PQ pool; however, their effects appeared insignificant. Overall, the differences of effects on the redox

status of PQ pools by different treatments between the short and the longer term were very small.

Photoacclimation Involves Changes of DNA-Binding Patterns in the *Lhcb1* Promoter

Within 2 h after an LL-acclimated culture was transferred to the HL condition, *Lhcb1* transcript levels decreased more than 10-fold (Fig. 4). The change in transcript abundance was concurrent with substantial decreases of LF1, LF2, and LF3 binding (Fig. 10A). *Lhcb1* transcript levels reached the lowest level within 6 h following the shift and then quickly recovered to 40% of the initial level. The rapid recovery of *Lhcb1* transcript abundance is concurrent with the dramatic increase of DNA-binding activities of LF1, LF2, and LF3 complexes (Figs. 4 and 10A). *Lhcb1* transcript levels reached a new, 40% lower, steady-state level 12 h following the shift. EMSA revealed that after 24 h, the binding activities of all four complexes were much lower than those in LL cells. The strong positive correlation between *Lhcb1* levels and the binding activities of LF1, LF2, and LF3 in the *Lhcb1* promoter region are also closely coupled in time, suggesting that these binding complexes enhance *Lhcb1* transcription. On the other hand, binding activities of LF factors (Fig. 10A) did not increase significantly while *Lhcb1*

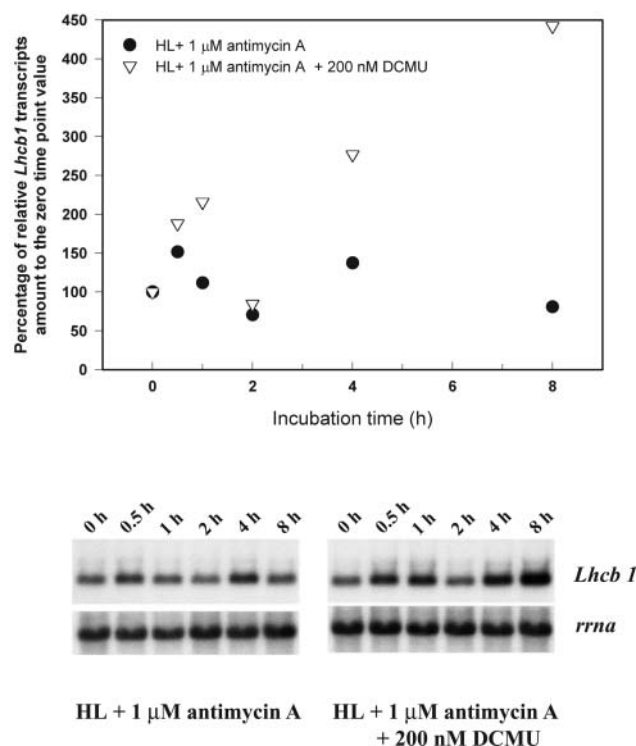**Figure 6.** Time course changes in *Lhcb1* and *rrna* transcript levels HL-acclimated cells following additions of 1 μM antimycin A or 1 μM antimycin A plus 200 nM DCMU. Top, Densitometric analysis of the time course changes in *Lhcb1* level, normalized to *rrna* level, as percentage to the zero time point value. Bottom, Northern-blot analysis of total cellular RNA probed sequentially with *Lhcb1* and *rrna*.

Figure 7. Time course changes in *Lhcb1* and *rrna* transcript levels in LL-acclimated cells following a shift to HL and 1.5 μM DBMIB additions to HL- and LL-acclimated cells. Top, Densitometric analysis of the time course changes in *Lhcb1* level, normalized to *rrna* level, as percentage to the zero time point value. Bottom, Northern-blot analysis of total cellular RNA probed sequentially with *Lhcb1* and *rrna*.
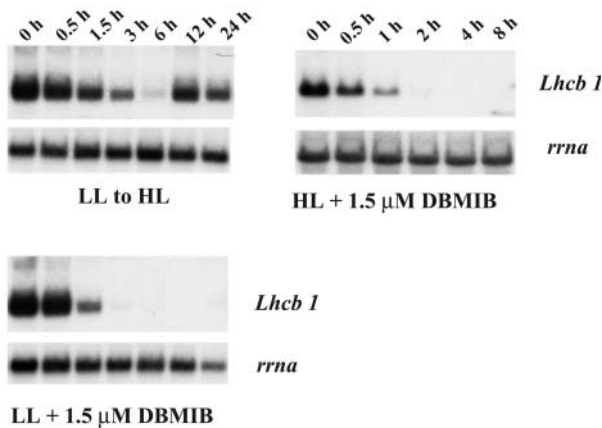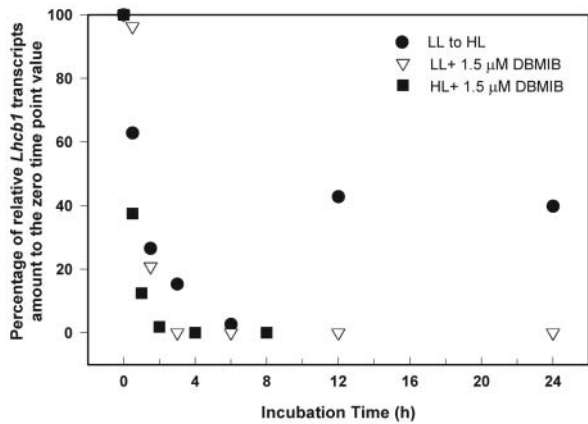
transcript abundance nearly tripled following an HL-to-LL shift (Fig. 3). Instead, the increased *Lhcb1* transcript abundance is coupled with a decreasing trend of the HLF-binding activity (Fig. 10A), suggesting a complicated role of HLF as a transcriptional repressor in regulating *Lhcb1* transcription.

Effects of Photosynthetic Inhibitors and Uncouplers on DNA-Binding Activities in the *Lhcb1* Promoter Region

We further investigated the relationship between *Lhcb1* transcript abundance and the DNA-binding patterns in the presence of PET inhibitors and uncouplers. The DNA-binding activities of LF1, LF2, and LF3 declined substantially 3 h after the DCMU addition to HL-acclimated cells, followed by dramatic increases 9 h later (Fig. 10B). The *Lhcb1* transcript abundance and the binding activities of LF1 and LF2 in the *Lhcb1* promoter region are not only positively related but also closely coupled in time, once again suggesting that LF factors may enhance *Lhcb1* transcription. The changes of bind-

ing activities of LF2 and LF3 following the addition of nigericin or valinomycin to HL cultures were similar but much smaller than those induced by DCMU (data not shown). While there were few changes in both the overall binding pattern and activities of individual complexes in cells treated by antimycin A alone, the binding activities of HLF, LF1, LF2, and LF3 were substantially enhanced in cells treated by both antimycin A and DCMU (Fig. 10C). The addition of 1.5 μM DBMIB to the LL culture led to an overall steady decrease of the binding activities of all complexes throughout the incubation, with HLF and LF1 activities virtually eliminated in the end, except that the binding of LF1 and LF2 increased at the 9-h time point (Fig. 10B). The overall patterns of changes in DNA-binding activities of all complexes are similar between those induced by the LL-to-HL shift and those by the DBMIB, yet their corresponding longer-term changes in *Lhcb1* transcript abundance differ (Fig. 7).

Effects of Thiol Group-Modifying Agents on DNA-Binding Complexes

Phosphatases, including calf intestine alkaline phosphatase, λ-protein phosphatase, and protein Tyr phosphatase, did not significantly affect in vitro binding activities of any of the complexes (data not shown).

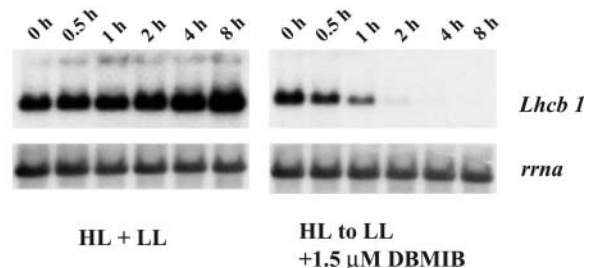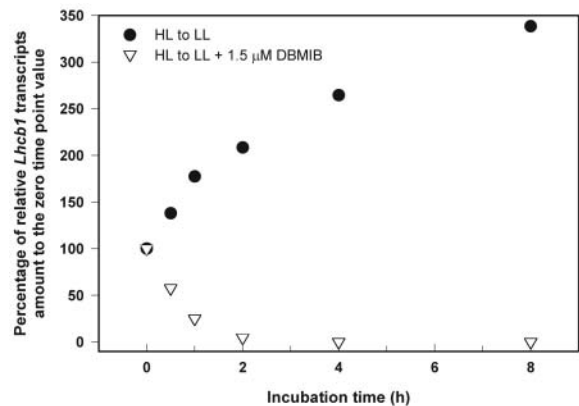**Figure 8.** Time course changes in *Lhcb1* and *rrna* transcript levels in HL-acclimated cells following a shift to LL with or without the presence of 1.5 μM DBMIB. Top, Densitometric analysis of the time course changes in *Lhcb1* level, normalized to *rrna* level, as percentage to the zero time point value. Bottom, Northern-blot analysis of total cellular RNA probed sequentially with *Lhcb1* and *rrna*.
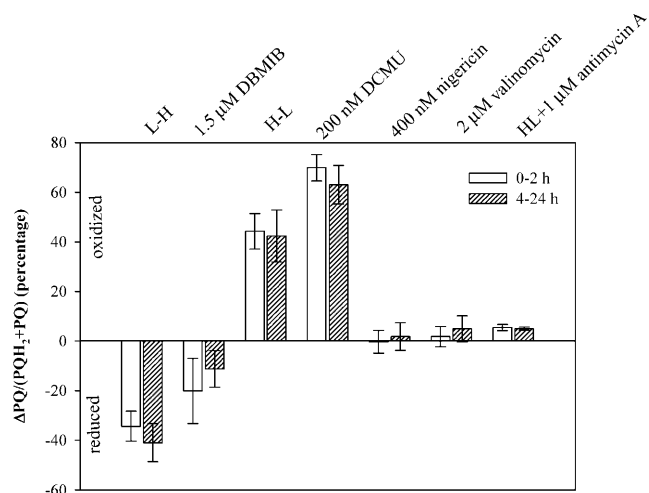
Figure 9. Short- and longer-term effects of various treatments on the PQ pool redox status. Error bars represented 1 × SD and n ranges from 3 to 12. Both HL and LL cultures were used in PET inhibitors and uncouplers treatments.

The effects of the thiol-oxidizing agent, azodicarboxylic acid bis-dimethylamide (diamide), and the thiol-modifying agent, *N*-ethyl maleimide (NEM), on the activities of the four DNA-binding complexes are shown in Figure 11. In vitro binding activities of HLF, LF1, LF2, and LF3 were all eliminated when whole-cell protein extracts were incubated with NEM before the addition of the oligo probes (Fig. 11). This was accompanied by the appearance of a new DNA-binding complex that migrated between LF1 and LF2. When NEM was added after the binding reaction was completed, however, the binding activity of HLF was enhanced while that of LF1 was reduced. Adding NEM after the completion of the binding reaction also resulted in less inhibition on the activities of other binding complexes than adding it prior to the binding reaction. The differences in sequence in which NEM was added affected DNA-binding activities, suggesting that the thiol group(s) in the complexes are not only directly involved in the binding in vitro, but can also enhance and reduce binding affinity of the HLF complex. While NEM dramatically inhibits in vitro binding activities of LF1, LF2, and LF3, diamide exhibits much less of an effect, especially if it is added after the binding reactions were completed (Fig. 11). However, diamide and NEM had a similar effect on HLF-binding activity. Taken together, the results of thiol group-modifying reagents treatment suggest that the assembly and binding of transcription factors to *Lhcb1* promoter is thiol group dependent.
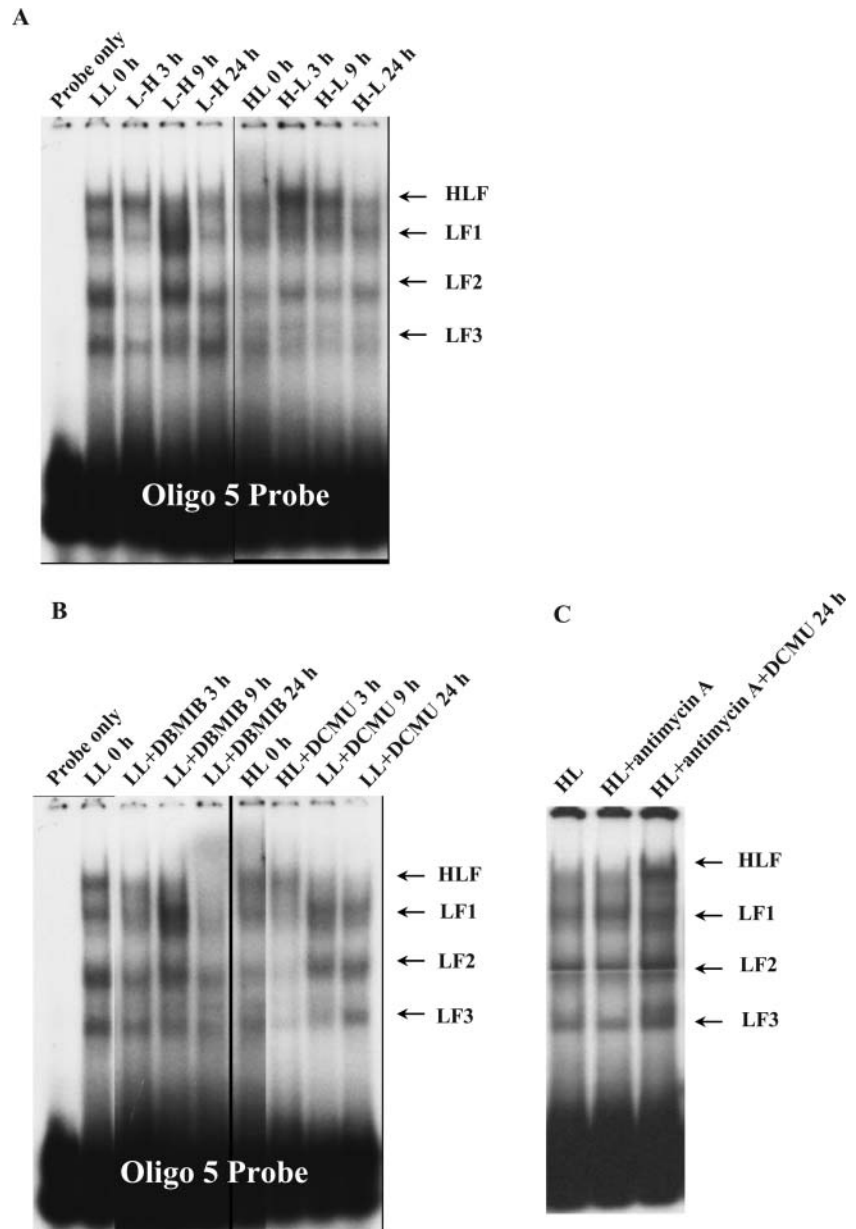
DISCUSSION

Chloroplastic Signals Affecting DNA-Binding Activities in the *Lhcb1* Promoter Region

Four DNA-binding complexes were detected when the *Lhcb1* promoter fragments were incubated with whole-cell protein extracts isolated from *D. tertiolecta*. The binding activity of the HLF complex is generally stronger in HL cells than in LL cells based on the experimental results conducted on exactly the same culture as the one used by Escoubas et al. (1995); however, from the results presented in this study, which used a different culture (with the same clonal designation), we did not observe the same pattern or a consistently inverse relationship between HLF-binding and *Lhcb1* mRNA levels, which suggests that the HLF complex may not be, by itself, a strong transcriptional repressor. In contrast, all three LF complexes are more abundant in cells grown under LL than those under HL. Their binding activities, when affected by light transitions or photosynthesis inhibitors, are not only positively related to the *Lhcb1* transcript abundance but also kinetically concordant with the latter (Fig. 10). Such results suggest that LF complexes may be part of a chloroplast-to-nucleus signal transduction pathway and function as transcriptional enhancers of *Lhcb1*. The dynamic changes of their binding activities, as well as the similarities of the binding patterns between those induced by the light transitions and those induced by various inhibitors, strongly suggest that they respond not only to the redox status of the PQ pool, but also to other chloroplast-derived signals that converge at the transcriptional level.

Our EMSA results indicate that larger binding complexes, such as HLF and LF1, were formed when the same protein sample was incubated with the nonoverlapping promoter fragments in the 180-bp region (Fig. 2). Escoubas et al. (1995) proposed that a G-box-like element located in the 180-bp region (from −265 to −254 bp) might be the binding site for the repressor-like HLF complex. However, the HLF complex binds to promoter regions lacking this element, clearly indicating that a G-box is not required for binding of this complex. It is possible that the 180-bp promoter region contains a number of motifs that are part of a multipartite cis-regulatory unit with repeating subunits, providing binding sites for several regulatory factors. The larger binding complexes, HLF and LF1, may be functional entities consisting of multiple DNA-binding factors whose protein-to-protein interactions determine their binding sites. It is also likely that the smaller DNA-binding complexes may represent the partial or intermediate versions of larger complexes with different regulatory functions. In addition, the coexistence of binding activities by all complexes in short promoter fragments strongly suggests that the binding sites of these complexes are closely located or even partially overlapped and/or all binding complexes share the same DNA-binding protein factor(s). The binding sites of LF2 and LF3 appear to be located in the −247 to −218-bp promoter region (Oligo 5) that contains both CACT and GGAA boxes (Figs. 1 and 2). The CACT box and GGAA box are uninterrupted only in Oligo 5, and the strongest binding of LF2, as well as the exclusive binding of LF3, was detected only in that region of the promoter.

Figure 10. EMSA of time course changes of DNA-binding activities in −247 to −218-bp region of *Lhcb1* promoter following the light transitions (A), additions of PET inhibitors (B), and antimycin A with or without the presence of DCMU (C). All protein samples were subjected to a single-step ammonium sulfate precipitation at 50% saturation level. LL 0 h, LL-acclimated culture prior to the light transition; L-H, LL culture transferred to HL condition; HL 0 h, HL-acclimated culture prior to light transition; H-L, HL culture transferred to LL conditions; LL+DBMIB, LL culture treated with 1.5 μM DBMIB; HL+DCMU, HL culture treated with 200 nM DCMU; HL+antimycin A, HL culture treated with 1 μM antimycin A for 8 h; HL+antimycin A and DCMU, HL culture treated with 1 μM of antimycin A and 200 nM of DCMU for 8 h.

Light-mediated transcription of *Lhc* genes in higher plants is regulated by the binding of regulatory proteins to upstream promoter regions (Argüello-Astorga and Herrera-Estrella, 1998). Detailed molecular studies of trans-acting factors and cis-acting elements have focused primarily on phytochrome-mediated signaling, whereas the regulation via chloroplast redox signaling remains poorly understood. While the *D. tertiolecta Lhcb1* promoter contains redundant control elements with identical or tightly linked light-responsive motifs (Fig. 1) similar to those in higher plants (Anderson and Kay, 1995; Terzaghi and Cashmore, 1995), the sequences of these tentative cis-elements differ from the GT-1 and G-boxes. In *C. reinhardtii*, the promoters of many light-responsive genes, including *Lhcb1*, also lack these cis-acting elements, which appear to be conserved in higher plants (Goldschmidt-Clermont and Rahire, 1986; Imbault et al., 1988; Hahn and Kück, 1999). The differences in promoter architecture suggest that the intermediate messenger, as well as possibly the mechanisms of light-regulation of photosynthetic gene expression, differ between higher plants and chlorophyte algae. However, the presence of a TCTAA site in *Lhcb* genes of *D. tertiolecta*, *C. reinhardtii*, and Arabidopsis suggests that some core chloroplastic signaling pathways may be highly conserved.
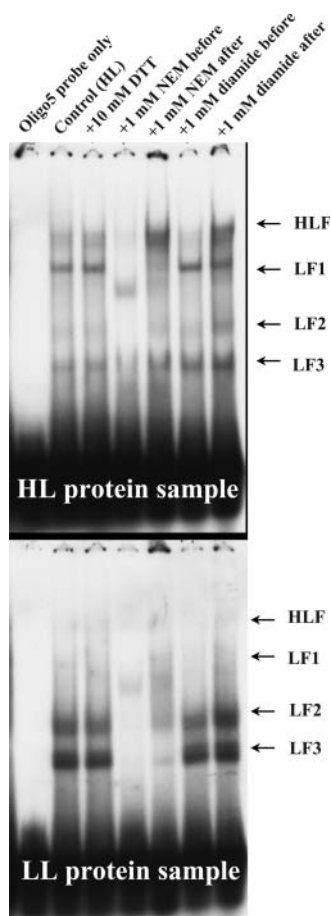
Figure 11. Effects of thiol group-modifying reagents on DNA-binding activities of various DNA-binding factors in *D. tertiolecta Lhcb1* promoter region from −247 to −218 bp. Whole-cell protein samples extracted from HL- and LL-acclimated cultures were subjected to a single-step ammonium sulfate precipitation at 50% saturation level.

Multiple Signals Involved in Transcriptional Regulation *Lhcb1*

The nuclear-localized *Lhcb* genes are tightly regulated by light intensity in both algae and higher plants. Escoubas et al. (1995) demonstrated that the transient increase in the transcriptional rate from a shift of HL to LL was the result of enhanced transcription. Similar conclusions have also been reached for *Dunaliella salina* (Masuda et al., 2002, 2003) and *C. reinhardtii* (Jasper et al., 1991; Durnford et al., 2003). Although plastid regulation of *Lhcb1* expression has been shown to occur at a transcriptional level (for review, see Gray et al., 2003), posttranscriptional regulatory mechanisms also may be involved (Hermsmeier et al., 1994; Durnford et al., 2003). Results of our northern blots revealed substantial and rapid decreases of the *Lhcb1* transcript levels in *D. tertiolecta* cells following the additions of DCMU (Fig. 3), DBMIB (Fig. 7), and nigericin and valinomycin (Fig. 5), as well as the shift to HL conditions (Fig. 4). These precipitous drops cannot be explained simply by a decrease in transcrip-

tion rate and the subsequent dilution of existing transcripts due to cell division. Although the stability of *Lhcb1* transcripts differs little between HL- and LL-acclimated cells (Escoubas et al., 1995; Masuda et al., 2002), our results strongly suggest that there is enhanced degradation of *Lhcb1* mRNA immediately following the addition of inhibitors/uncouplers or a shift to the HL conditions. This phenomenon was reported in *C. reinhardtii* (Durnford et al., 2003) and further supported in a study on *D. salina* in which a significant decrease in the half-life of *Lhcb* transcripts was detected immediately following an LL-to-HL shift (Masuda et al., 2003). The PQ pool redox state has also been suggested to regulate mRNA stability of the nuclear-encoded *PetE* gene in higher plants (Sullivan and Gray, 2002). Hence, mRNA degradation may also contribute to the short-term down-regulation of *Lhcb1* expression in *D. tertiolecta*.

On longer time scales however, the redox state of the PQ pool clearly regulates the transcription of *Lhcb1* gene. DBMIB always results in both short-term and longer-term decreases in *Lhcb1* transcript levels in both HL and LL culture (Fig. 7) and prevented increases in *Lhcb1* transcription during HL-to-LL transitions (Fig. 8). Transient fluorescence measurements indicated that in all DBMIB treatments, the PQ pool was rapidly reduced within the first 1 to 2 h before some recovery (Fig. 9), suggesting that the reduced PQ pool and/or cytochrome $b_6 f$ are among the chloroplastic signals responsible for down-regulation of *Lhcb1* transcription. DCMU, which always led to an oxidized PQ pool, induced increases in both *Lhcb1* transcript levels and Chl synthesis 6 to 8 h following the addition of the inhibitor (Fig. 3). Although the short-term decline in *Lhcb1* transcript abundance immediately following DCMU addition could be mediated by a different chloroplastic signal and/or changes in transcript stability, the longer-term DCMU effects on *Lhcb1* transcription and Chl synthesis phenotypically resemble those by an HL-to-LL shift. These results suggest that a signal initiated from the oxidized PQ pool regulates longer-term photoacclimative responses.

There is, however, strong evidence that the redox state of the PQ pool is not the only photosynthetic signal that regulates *Lhcb1* transcription, especially in the short term. Despite the fact that DCMU additions always resulted in a rapidly oxidized PQ pool (Fig. 9), both *Lhcb1* transcript levels (Fig. 3) and Chl synthesis decreased substantially within the first 4 to 6 h (data not shown). Furthermore, when added to an LL culture prior to a shift to HL, DCMU failed to prevent decreases in both *Lhcb1* transcript level (Fig. 4) and Chl synthesis, although the inhibitor prevented the PQ pool from being reduced by the elevated light intensity. DCMU's inability to prevent *Lhcb* genes from being down-regulated by HL was also reported in *C. reinhardtii* (Teramoto et al., 2002). Together, these results unequivocally demonstrate that the PQ pool redox status is not the sole signal involved in regulating *Lhcb1* transcription in the short term; either

the signal from the PQ redox state is overwritten by the other signal(s) or the PQ pool signal belongs to a longer-term chloroplastic signaling pathway, or both. Changes in ΔpH have been suggested as a rapid and powerful photosynthetic signal initiator (Horton et al., 1996; Wilson and Huner, 2000). It is conceivable that DCMU addition resulted in an immediate decrease in membrane potential, manifested by ΔpH, $\Delta\psi$, and ATP production, triggering signals to rapidly down-regulate *Lhcb1* transcription. While such negative signals may overwrite the up-regulation redox signals from the oxidized PQ pool, the balance is reversed in the longer term as the redox state of the latter electron carrier becomes dominant.

Like DCMU, addition of nigericin and valinomycin to LL and HL cultures resulted in sizable decreases in *Lhcb1* transcript levels within the first 2 h, which then recovered and climbed to a new steady-state level at about 50% higher than the previous level (Fig. 5). Since nigericin uncouples photophosphorylation by dissipating ΔpH while valinomycin eliminates $\Delta\psi$, the similarities of their impact on *Lhcb1* transcription clearly indicate that their regulation of *Lhcb1* transcription may be at least partially achieved via photophosphorylation. The initial down-regulation of *Lhcb1* expression may be resulted from an immediate decrease of ΔpH/ATP production caused by the uncouplers. On the longer time scale, however, the uncouplers facilitate PQH_2 oxidation (Rees et al., 1989; Hope et al., 1994), resulting in a more oxidized cytochrome b_6f complex, which appears to up-regulate *Lhcb1* transcription.

The role of ATP and/or ΔpH as possible chloroplast signal initiators is further supported by the experiments using antimycin A with or without DCMU. Our fluorescence measurements indicate that antimycin A slightly enhanced the oxidation of the PQ pool (Fig. 9); however, by itself, it had little overall effect on *Lhcb1* transcript levels in HL cells (Fig. 6). It is clear that either antimycin A did not have any impact on mitochondria at the concentration used in this experiment or such impact did not affect *Lhcb1* transcript abundance. On the contrary, when both DCMU and antimycin A were added, *Lhcb1* transcript level rose rapidly from the beginning and quadrupled within 8 h (Fig. 6). The strong, positive, short-term impact of the combinational treatment by DCMU and antimycin A on *Lhcb1* transcript levels suggests that, when linear electron transfer is inhibited, cyclic electron flow around PSI may play an important role in maintaining redox poise of cytochrome b_6f as well as transthylakoid membrane potential. In the presence of DCMU, inhibition by antimycin A on PSI cyclic electron flow further down-regulates the ΔpH, thus leading to an oxidation state of the PQ pool. We hypothesize that the effect may be sensed via the redox state of cytochrome b_6f complex, as this protein complex has direct access to both stromal and luminal sides of the thylakoid membrane (Kurisu et al., 2003). Indeed, a phenotype of photosynthetically competent mutants of Chla-

mydomas, which have impaired binding of PQH_2 to cytochrome b_6f is a chronic down-regulation of *Lhcb* expression (Falkowski and Chen, 2003). There is growing evidence that the cytochrome b_6f complex, the redox status of which is determined not only by linear electron transport through the PQ pool but also by cyclic electron transfer around PSI, may play a critical role in the regulation of *Lhcb1* expression via LHCBII kinase(s) (Pursiheimo et al., 2001; Yang et al., 2001; Kurisu et al., 2003; Zer and Ohad, 2003). Although the molecular mechanism through which the redox state of the PQ pool is transduced to the kinase is unknown, the implication of the quinol binding site, Q_o, of the cytochrome b_6f complex has been made both in vivo in *C. reinhardtii* (Zito et al.; 1999, Finazzi et al., 2001) and in thylakoid preparations from spinach (Vener et al., 1995, 1997).

While the combined effects of DCMU and antimycin A resemble those by DBMIB, there is one major difference between the two scenarios: the PQ pool and cytochrome b_6f in DBMIB-treated cells are much more reduced than those in cells treated by both DCMU and antimycin A (Fig. 9). Their opposite effects on *Lhcb1* transcription clearly point out the importance of the redox status of the PQ pool as a chloroplastic signal, as well as the notion that different signals regulate nuclear *Lhcb1* transcription in a cooperative fashion. However, the effect of DCMU plus antimycin A largely resembles the acclimation to LL in terms of the simultaneous reduction of ΔpH/ATP and the oxidation of the PQ pool and potentially cytochrome b_6f complex. The similar time course changes of *Lhcb1* transcript abundance between the two scenarios suggest that a reduction in both linear intersystem electron flow and cyclic electron flow around PSI stimulates *Lhcb1* transcription.

Role of Thiol Groups in DNA-Binding Activities

Our EMSA results revealed that the binding affinity of all four complexes clearly involves thiol group modification in vitro (Fig. 11), a phenomenon that has been reported for several transcription factors in higher plants and animal cells (for review, see Sun and Oberley, 1996). Based on the PET inhibitor and uncoupler results, it is very unlikely that such modifications occur in vivo via the ferredoxin/thioredoxin redox couple on the acceptor side of PSI (e.g. Danon and Mayfield, 1994). Escoubas et al. (1995) suggested, based on the phosphatase inhibitor studies in vivo, that a phosphorylation cascade, potentially coupled to the PQ redox-sensitive kinase/phosphatase system(s), relayed chloroplastic signals to the nucleus. Our results suggest, however, that the DNA-binding activities of the complexes examined are not altered by phosphatases (data not shown), and therefore the signal transduction pathway must involve additional mechanisms other than a phosphorylation cascade (Escoubas et al., 1995; Carlberg et al., 2003). Unless the binding factors can be translocated across the plastid envelope

membrane, thiol group modification must occur in the cyotsol. Interestingly, thiol groups are highly sensitive to H_2O_2. Hydrogen peroxide is produced inside the chloroplasts of cells that are exposed to excessive light intensities, and it is readily diffusible out of chloroplasts. The H_2O_2 molecule constitutes a dominant chloroplast-derived reactive oxygen species, and it has been implicated in the signaling of excessive light intensity and regulating the expression of nuclear genes (Mullineaux and Karpinski; 2002; Wilson et al., 2003).

CONCLUSION

Although the lack of a transformation system limits our ability to modify the promoter region of *Lhcb1* in *D. tertiolecta*, our results reveal, for the first time to our knowledge, three complexes that bind to the *Lhcb1* promoter are closely coupled to the chloroplastic signals and are correlated with enhanced transcription of the gene in vivo. Sequence analysis of the targeted *Lhcb1* promoter region revealed multiple and repetitive sequences as possible binding sites for these DNA-binding complexes. Results from our time course study of *Lhcb1* transcription affected by light transitions and various photosynthesis inhibitors indicate the nuclear *Lhcb1* gene expression may be cooperatively regulated by multiple chloroplastic signals. Specifically, our results suggest that the short-term expression of *Lhcb1* is primarily regulated by transthylakoid membrane potential signals triggered by $\Delta pH/ATP$ pool, while the longer-term expression is primarily regulated by the redox signals initiated from the PQ pool.

MATERIALS AND METHODS

Materials

Restriction, modifying, and amplifying enzymes were purchased mainly from Promega (Madison, WI) and used according to vendor's instructions. Chemicals for making medium and various buffer solutions and reagents were purchased from Fisher Scientific (Hampton, NH) and Sigma (St. Louis). $[\alpha\text{-}^{32}P]dATP$ and $[\gamma\text{-}^{32}P]ATP$ (3,000 Ci/mmol) were purchased from Perkin-Elmer Life and Analytical Sciences (Boston) and AP Biotech (Piscataway, NJ). Primers and oligo DNA were synthesized and purified by Integrated DNA Technologies (Coralville, IA).

Cell Cultures

Dunaliella tertiolecta (clone DUN, Provasoli-Guillard Culture Collection, West Boothbay Harbor, ME) was grown in 20-L-size batch culture using filtered off-shore North Atlantic seawater amended with f/2 nutrients (Guillard and Ryther, 1962) and bubbled with air. Cultures were grown between 24°C and 28°C in an EGC growth chamber, under continuous light (cool-white) and maintained optically thin ($<1\times10^6$ cells/mL) to avoid self shading. Cultures were routinely grown between 1,000 and 1,600 μmol quanta $m^{-2}\ s^{-1}$ (HL) or approximately 80 to 120 μmol quanta $m^{-2}\ s^{-1}$ (LL). Light intensity was controlled by a combination of the number of fluorescent tubes used as light source and through the use of black insect screening as a neutral-density filter. Cells were typically acclimated to a particular light intensity for 60 to 72 h prior to initiating experiments to ensure the cells were in the expected physiological state. Chl and cell density were analyzed as previously described (Escoubas et al., 1995).

RNA Extraction and Northern Blots

At each sampling point, 90 to 150 mL culture ($3-10\times10^7$ cells) was harvested by centrifugation. The cells were immediately lysed in an RNA extraction buffer (1.2% [w/v] SDS, 30 mM EDTA, 50 mM Tris-HCl, pH 8.0, 220 mM NaCl, and 50 mM β-mercaptoethanol). The subsequent RNA processing was carried out following commonly used protocols with minor modification (Sambrook et al., 1989).

Northern-blot analysis was performed to determine the time course abundance of *Lhcb1* (previously known as *cab1*; LaRoche et al., 1991) transcripts of *D. tertiolecta* cultures grown under different conditions. Northern-blot analysis was carried out following widely used protocols (Sambrook et al., 1989) with minor modifications. An equal amount of total RNA (approximately 5–10 μg) was fractionated by electrophoresis on a 1% [w/v] agarose gel with 1 M formaldehyde. The intensities of ethidium bromide-stained rRNA bands were visually examined to ensure that equal amounts of total RNA were loaded for all samples. The RNA was then transferred to a charged nylon membrane (Nytran, Schleicher & Schuell, Keene, NH) and crosslinked to the membrane using a Stratalinker (Stratagen, La Jolla, CA). ^{32}P-labeled DNA probes were hybridized to the northern blots overnight at 42°C in the PerfectHyb solution (Ambion, Austin, TX). The blots were then washed twice with 2× SSC (0.3 M NaCl and 0.03 M sodium citrate) and 0.1% [w/v] SDS for 30 min at 42°C, rinsed with 0.2× SSC and 0.1% [w/v] SDS, and exposed to BioMax MR/MS films (Eastman-Kodak, Rochester, NY). A *D. tertiolecta Lhcb1*-specific fragment was amplified from the 3′-untranslated region of a *D. tertiolecta Lhcb1* cDNA clone (cDNA4) with specific primers (forward primer, 5′-TAAATGCTGAAATGCAG-3′ and reverse primer, 5′-TAAGGCCAGTAA-GAAT-3′). The fragment was labeled with $[\alpha\text{-}^{32}P]dATP$ using a random-primed labeling kit (Promega). To account for variations in total RNA loading, *Lhcb1* signals were standardized to rRNA levels determined by hybridization of a *Chlamydomonas reinhardtii* rDNA probe (Clone P92) to the same northern blots whose *Lhcb1* probes were stripped by incubating in stripping solution (0.2× SSC, 0.2% [w/v] SDS) at 95°C for 20 min followed at 65°C overnight. For densitometric analysis of signals, original northern autoradiograms were scanned into a computer using an Epson 1680 Pro Scanner (1,600×3,200 dpi hardware resolution, 48-bit color with 3.6 Dmax optical density, Epson America, Long Beach, CA). The public domain NIH Image program (developed at the United States National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image) was used to perform densitometric analysis of labeled bands. To minimize artifacts introduced during the northern assay and subsequent quantitative analysis, we compare only samples on the same gel/same batch of gels and plotted them on a relative scale (percentage of zero point value).

Protein Extraction and Measurement

Culture harvest and protein extraction were conducted essentially as previously described (Escoubas et al., 1995). A modified high-salt extraction buffer (25 mM HEPES-KOH, pH 8.0, 0.5 M KCl, 1 mM EDTA, 20 mM β-mercaptoethanol, 0.2 M Suc, and 1 mM magnesium acetate) was used. The cellular homogenate, clarified by ultracentrifugation at 200,000g for 30 min at 4°C, is routinely precipitated with $(NH_4)_2SO_4$ at 50% [w/v] saturation. The precipitated proteins are collected by ultracentrifugation at 35,000g for 20 min at 4°C. The protein pellet is resuspended in a storage buffer (25 mM HEPES-KOH pH 7.9, 40 mM KCl, 5 mM β-mercaptoethanol, 1 mM EDTA, and 10% [v/v] glycerol), and desalted using Econo-Pac 10DG columns packed with Bio-Gel P-6G gel (Bio-Rad Laboratories, Hercules, CA). The protein concentration is determined in duplicates with the BCA assay kit (Pierce Chemical, Rockford, IL). A protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 5 mM ε-amino-*n*-caproic acid, 1 mM benzamidine chloride, 100 μM sodium metabisulfite, 1 mg/mL aporotinin, and 1× General Protease Inhibitor Cocktails by Sigma) was added to both extraction and storage buffers.

Preparation of *Lhcb1* Upstream Fragments for EMSA

Our entire study of DNA-binding activities in *D. tertiolecta Lhcb1* promoter was centered on a 180 bp region (−367 to −188 bp). Six 30-bp long double-stranded oligo DNA (Oligo 1–6) were constructed by annealing six pairs of commercially synthesized single-stranded oligos (Integrated DNA Technologies), which sequentially represent both strands of the 180-bp upstream region of *Lhcb1*. These oligo DNA were isolated via agarose gel electrophoresis and subsequently purified using Centrex MF-0.4 micro-centrifuge filters (Schleicher & Schuell) and Microcon-10 devices (Millipore, Bedford, MA).

The purified oligo DNA were quantified and used as either labeled probes or unlabeled competitors in EMSA. The locations and sizes of the oligo DNA are presented in Figure 1.

EMSA

EMSA was used to study DNA-binding activities in the *Lhcb1* upstream region of *D. tertiolecta* cultures grown under different conditions. In all EMSA, the labeled oligo DNA probes were added at over-saturating level so that the DNA protein-binding activities were limited only by the amount of the DNA-binding proteins presented in the binding mixtures. The binding reaction and the EMSA were performed based on previously described protocols with minor modifications (Escoubas et al., 1995). For competition EMSA, the competitors were added together with the probes. Treatment with the thiol-modifying agents was carried out essentially as described in Jiang et al. (1997). Treatment with the thiol-oxidizing agent diamide (Sigma) was conducted by incubating crude protein extract in the binding buffer for 1 h at 37°C in the presence of 1 mM diamide either before or after a 40 min incubation with labeled promoter fragments. The same procedures were followed for treatment with 1 mM thiol group modifying agent NEM (Sigma). To ensure a proper comparison, the same amount of total protein samples extracted from different cultures from the same experiment was used in each binding reaction. For all physiological experiments, EMSA were done using protein samples obtained from a single 50% [w/v] $(NH_4)_2SO_4$ cut. The results from the incubation of the protein samples with Oligo 5 are presented since the activities of all four complexes could be clearly detected only with this probe.

Light Transition Studies

The light transition studies were conducted to examine the changes of DNA-binding activities in the *Lhcb1* upstream region in responding to the change of light intensities. Two one-step transition experiments from LL (70–100 μmol quanta m^{-2} s^{-1}) to HL (1,200–1,400 μmol quanta m^{-2} s^{-1}) and from HL-to-LL transitions were carried out. For each transition experiment, 20-L batch culture of *D. tertiolecta* was grown and acclimated to the initial light intensity. At the beginning of the experiment, the culture was equally divided into two 10-L subcultures, with one remaining under the initial light conditions while the other was transferred to a different light condition. The incubation lasted for 8 h following the light shift. Samples for Chl, cell density, and RNA analysis were taken at the mid and end point of the incubation. The biomass from each subculture was harvested for protein extraction at the end of the experiment.

Inhibitor Studies

Results from preliminary experiments based on measurements of variable Chl fluorescence, Chl, and cell density were used to choose the proper concentrations for each inhibitor, ensuring their effective inhibition on photosynthesis while still allowing cells to grow. Stock solutions of photosynthetic electron transfer inhibitors DCMU and DBMIB were made in dimethyl sulfoxide. Stock solutions of photophosphorylation uncouplers nigericin and valinomycin were made in ethanol, as was the PSI cyclic electron transfer inhibitor antimycin A. Based on the dose response analyses, we used the following concentration for each inhibitor: DCMU, 200 nM; DBMIB, 1 μM initial with repletion of 250 nM every 4 to 5 h to remedy the labile nature of DBMIB; nigericin, 400 nM; valinomycin, 2 μM; and antimycin A, 1 μM. At the zero time of each inhibitor experiment, batch cultures of *D. tertiolecta* acclimated to HL or LL conditions were divided into the treatment subculture that receives the inhibitor and the control subculture that receives the solvent (in which the inhibitors stock solutions were made) with the level identical to what was added to the treatment subculture. Variable fluorescence of each subculture was monitored frequently throughout the entire incubation using a pulse amplitude-modulated fluorometer to ensure the effectiveness of the inhibitors on photosynthetic electron transfer. Samples for Chl, cell density, RNA, and protein were taken according to predetermined time intervals.

For the combination treatments involving both light transitions and inhibitor addition, the inhibitors were added to the cultures just prior to the light shift.

FRR Fluorometry

The photosynthetic performance and redox status of the PQ pool were assessed using the FRR fluorometer (Kolber et al., 1998) with a modified detection unit (large area avalanche photodiode detector, Advanced Photonix, Camarillo, CA). The instrument generates a train of short (0.6 μs) blue (470 nm) flashlets in the microsecond-to-millisecond timescale. Electron transport elicited by these light pulses induces transient changes in Chl *a* fluorescence emission reflecting redox and light acclimation status of the photosynthetic machinery. The FRR Chl fluorescence transient was recorded in the dark, and the fluorescence parameters F_v/F_m and σ_{470nm} (functional cross section of photosynthetic units) were determined as described previously (Kolber et al., 1998). The cells in the measuring chamber were then exposed to continuous background irradiance simulating the light conditions used in the experiments for 1 min, and the second FRR fluorescence transient was recorded. The oxidation status of PQ pool was estimated as $(F'_m - F_s)F_m/F'_m(F_m - F_0)$, where F_s is the steady-state fluorescence intensity and F'_m is the maximum fluorescence in the light.

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