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Chloroplast Response in *Dunaliella salina* to Irradiance Stress[1]

Effect on Thylakoid Membrane Protein Assembly and Function

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The chloroplast response in the green alga *Dunaliella salina* to irradiance stress was investigated. Cells were grown under low light (LL) at 100 μmol photons m^{-2} s^{-1} or high light (HL) at 2000 μmol photons m^{-2} s^{-1} incident intensity. LL-grown cells had a low chlorophyll (Chl) *a/b* ratio, an abundance of light-harvesting complex II proteins (LHC-II), and a large Chl antenna size. HL-grown cells had a higher Chl *a/b* ratio, relatively fewer LHC-II, and a small Chl antenna size. The more abundant higher molecular mass subunits of the LHC-II (approximately 31 kD) were selectively depleted from the thylakoid membrane of HL-grown cells. Light-shift experiments defined the kinetics of change in the subunit composition of the LHC-II and suggested distinct mechanisms in the acclimation of thylakoids to HL or LL conditions. The results showed that irradiance exerts a differential regulation on the expression of various *Lhcb* genes. The specific polyclonal antibodies used in this work, raised against the purified LHC-II, cross-reacted with a polypeptide of approximately 20 kD in HL-grown samples. In this work we examined the dynamics of induction of this novel protein and discuss its function in terms of a chloroplast response to the level of irradiance.

LHC-II contains a minimum of six polypeptide subunits with molecular masses in the 25- to 31-kD region. These proteins are encoded by a nuclear family of genes now named *Lhcb* (formerly *cab*) (Jansson et al., 1992). The genes of this family show a high degree of homology to each other and to a lesser degree to the *Lhca* genes, which code for the subunit proteins of the LHC-I. Both *Lhca* and *Lhcb* genes are members of a larger superfamily of genes, which includes the genes coding for the so-called ELIPs (Grimm et al., 1989), the Cbr protein (Lers et al., 1991), and the recently identified *PsbS* gene (Kim et al., 1992; Wedel et al., 1992). All members of this superfamily appear to be nuclear-encoded genes. The mature products of these genes all contain highly conserved transmembrane helices and display varying degrees of immunological cross-reactivity with one another. Their amino acid sequences diverge sharply, however, in their amino-terminal regions. There also appears to be considerable difference in the Chl-carotene-xanthophyll-binding properties of the various apoproteins.

The level of irradiance during plant growth modulates the size and composition of the light-harvesting antenna of the photosystems (Anderson, 1986; Melis, 1991). In general, LL intensity promotes a larger Chl antenna size for both PSI and PSII (larger photosynthetic unit size). HL conditions elicit a smaller Chl antenna size. This adjustment in the Chl antenna size of the photosystems comes about because of changes in the size of the auxiliary LHC-II and LHC-I (Leong and Anderson, 1984; Larsson et al., 1987; Sukenik et al., 1988; Smith et al., 1990). Mechanistically, these changes are implemented through the association of variable amounts of Lhcb proteins with the light-harvesting antenna of PSII (Larsson et al., 1987; Morrissey et al., 1989; Mawson et al., 1994). The response appears to be highly conserved in all photosynthetic organisms examined. However, the amplitude of the response differs significantly among different species. Green algae display a significantly greater amplitude of this response than higher plants, making them a better model organism in such studies. Recent work by LaRoche et al. (1991) suggested that changes in the abundance of LHC-II are preceded by changes in the abundance of the mRNA coding for these proteins. The regulation of these phenomena at the molecular and membrane levels is currently unknown.

When the green alga *Dunaliella salina* is grown at a high irradiance (2000 μmol photons m^{-2} s^{-1}), a number of changes occur in the structural and functional organization of its thylakoid membranes, including a decrease in Chl/cell, an increase in the Chl *a*/Chl *b* ratio, and a decrease in the LHC-II antenna size (Smith et al., 1990). It is also known that variations in the level of irradiance during cell growth bring about changes in the expression of ELIP genes in pea (Adamska et al., 1993) and of *Cbr* genes in *Dunaliella bardawil* (Levy et al., 1992). The relationship between light-harvesting antenna size in chloroplasts and the regulated

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Abbreviations: Cbr, carotene biosynthesis related; ELIP, early light-inducible proteins; HL, high light; LHC, light-harvesting complex; LHC-I, Chl *a-b* light-harvesting complex of photosystem I; LHC-II, Chl *a-b* light-harvesting complex of photosystem II; LL, low light; S_{LL} and S_{HL}, slopes of the logarithmically linear growth phase of LL and HL cells, respectively.

expression of ELIP and/or *Cbr* genes has not been addressed.

In this report, we examine the dynamics of LHC-II assembly as a function of irradiance. We used light-shift experiments in which *D. salina* cultures were switched from LL to HL conditions and vice versa. This approach helps us to identify the LHC-II subunits that change the most in response to irradiance. Furthermore, we describe the presence and dynamics of an approximately 20-kD novel protein found in association with the chloroplast in the green alga *D. salina*. This protein is immunochemically recognized by polyclonal antibodies specific for the LHC-II and appears to be induced under conditions of irradiance stress.

MATERIALS AND METHODS

Cell Growth Conditions

Dunaliella salina cultures were grown in an artificial hypersaline medium similar to that used by Pick et al. (1986) containing 1.5 M NaCl, 5 mM $MgSO_4$, 0.3 mM $CaCl_2$, 0.1 mM KH_2PO_4, 20 mM EDTA, 2 mM $FeCl_3$, 5 mM NH_4Cl, and 40 mM Tris-HCl, pH 7.5, and supplemented with a mixture of micronutrients. Carbon was supplied as $NaHCO_3$ in the growth medium at an initial concentration of 25 mM. Cultures were grown in flat bottles (optical pathlength = 3 cm) at 30°C under illumination at 100 μmol photons m^{-2} s^{-1} (LL) or at 2000 μmol photons m^{-2} s^{-1} (HL). Care was exercised, by means of shaking and by the use of reflectors, to ensure as uniform illumination to the culture as possible. Cells were grown to the late ln phase ($\ln A_{678} \cong -2.0$ for LL-grown cultures, $\ln A_{678} \cong -3.3$ for HL-grown cultures) and treatments (transfer to different irradiance conditions and/or addition of antibiotics) were made. In some experiments, aliquots of cells were harvested at different stages of growth, e.g. from the early logarithmic phase to the late stationary phase of culture development.

The A_{678} of the cell suspension ($\ln A_{678}$) was measured at room temperature using the technique of Shibata (1958). An Aminco (Urbana, IL) DW 2a spectrophotometer was used with a 3-nm half-band width and opal glass cuvettes of 1 cm optical pathlength placed directly in front of the photomultiplier tube to minimize light-scattering effects.

Thylakoid Membrane Isolation

Cells were harvested by centrifugation at 3000*g* for 3 min at 4°C. Pellets were resuspended in 0.5 mL of fresh growth medium and stored frozen at −20°C until all samples were ready for processing. Samples were thawed on ice and diluted with sonication buffer containing 50 mM Tris-HCl, 0.1% sodium ascorbate, 1 mM aminocaproic acid, and 1 mM aminobenzamidine, pH 8.5. Cells were disrupted by sonication for 30 s in a Branson (Danbury, CT) Sonifier (Cell Disruptor 200) operated in the pulsed mode with a 50% duty cycle and an output power setting of 5. Unbroken cells and other large cell fragments were removed by centrifugation at 3000*g* for 3 min at 4°C. The supernatant was then centrifuged at 46,000*g* for 30 min at 4°C. The thylakoid membrane pellet was resuspended in 200 mM Tris, 20% glycerol, pH 6.6, and Chl concentrations were determined in 80% acetone according to the method of Arnon (1949).

Membranes were solubilized in buffer containing 75 mM Tris-HCl, 3.5% SDS, 10% glycerol, and 4 M urea, pH 6.8, and incubated at room temperature for 15 min. This approach (denaturation of thylakoid membrane proteins at room temperature by SDS-urea) was chosen to avoid aggregation of the hydrophobic light-harvesting polypeptides. In our experience, heat denaturation resulted in the formation of high mol wt aggregates that appeared either as a smear in the stacking gel and upper portion of the running gel or as a high mol wt band of protein immobilized at the stacking/running gel interface. Prior to electrophoresis, samples were centrifuged in a microcentrifuge for 5 min to remove unsolubilized material. β-Mercaptoethanol was added to samples to give a final concentration of 10% and samples were diluted accordingly to yield equal Chl concentrations. Samples were stored on ice until used or otherwise stored at −80°C.

Thylakoid Membrane Protein Analysis

Thylakoid membrane proteins were resolved by SDS-PAGE using the discontinuous buffer system of Laemmli (1970) with 15% acrylamide and 0.41% bis-acrylamide, with or without 4 M urea. The stacking gel contained 4.5% acrylamide (and 1 M urea, as appropriate). The gel lanes were loaded with 2 to 4 nmol Chl (*a* plus *b*) for SDS-PAGE and immunoblot analysis unless otherwise indicated. Electrophoresis on 0.15- × 14- × 16-cm slab gels was performed at 2°C at a constant current of 8 mA for 18 h. Gels were stained with 0.1% Coomassie brilliant blue R for protein visualization.

Immunochemical Analysis

Identification of LHC (*Lhcb* gene) polypeptides was accomplished with immunoblot analysis using specific polyclonal antibodies raised in this laboratory in rabbits against proteins of LHC-II. Antigens from spinach were obtained from resolved membranes of the grana partition regions (the so-called BBY particles) (Berthold et al., 1981) following a modification of the method of Burke (1978). The isolated LHC-II was precipitated upon incubation with $MgCl_2$ and washed twice (Burke, 1978). Its purity was tested by SDS-PAGE analysis. The antigen, containing the isolated LHC-II in its native form, was injected subcutaneously in rabbits. This approach prevented antigen contamination by other thylakoid membrane proteins and resulted in the formation of monospecific polyclonal antibodies (Melis, 1992).

Electrophoretic transfer of the SDS-PAGE resolved *D. salina* thylakoid membrane polypeptides to nitrocellulose, and the subsequent incubations with the antibodies and with alkaline phosphate-conjugated antibodies were performed as described previously (Smith et al., 1990). Cross-reaction was quantitated by scanning the nitrocellulose membranes with an LKB-Pharmacia XL laser densitometer.

RESULTS

Cell Growth and Chl Content under LL and HL Conditions

D. salina was grown under uniform illumination either at 100 μmol photons m^{-2} s^{-1} (LL conditions) or at 2000 μmol photons m^{-2} s^{-1} (HL conditions). Cell growth was monitored by measuring ln A_{678}. We determined that the Chl/cell ratio remained fairly constant under continuous LL or HL growth conditions (Naus and Melis, 1991). Therefore, the ln A_{678}, when plotted as a function of time after culture inoculation (Fig. 1), provides a measure of Chl accumulation and of cell growth under LL and HL conditions. Figure 1 shows a logarithmically linear phase of growth and a gradually slower phase, followed by the stationary phase.

Rates of growth in the LL and HL culture were estimated from the growth curves, defined by the slope at the logarithmically linear phase. Our analyses indicated an S_{LL} = 1.3 ± 0.2 d^{-1} and an S_{HL} = 0.9 ± 0.2 d^{-1}, suggesting a slightly faster cell growth under LL than HL conditions. It is known that *D. salina*, grown under optimal light intensities, is capable of higher rates of growth at the logarithmic-linear phase, approaching slopes of about 5 d^{-1} at an incident intensity of about 500 μmol photons m^{-2} s^{-1} (Smith et al., 1990). Thus, under our LL conditions, an S_{LL} = 1.3 d^{-1} suggests growth limited by the intensity of illumination. Under our HL conditions, an S_{HL} = 0.9 d^{-1} indicates irradiance stress and a chronic photoinhibition condition (Kim et al., 1993) that limit photosynthesis and cell growth.

LL and HL cultures reached approximately the same cell density (approximately 2 × 10^6 cells mL^{-1}) in the stationary phase (Kim et al., 1993). However, they differed significantly in the Chl *a*/Chl *b* ratio (approximately 4:1 for the LL cells and approximately 17:1 for the HL cells). They also differed in the Chl/cell ratio, a fact that is illustrated in Figure 1 by the significantly lower ln A_{678} values in the HL culture relative to the LL culture at all comparable stages of growth. In the stationary phase, the LL-grown culture contained about 4 × 10^{-13} mol of Chl/cell, whereas the HL-grown cells reached approximately 0.8 × 10^{-13} mol of Chl/cell (ln A_{678} = −0.8 versus ln A_{678} = −2.3). These quantitative differences are consistent with earlier findings from this laboratory (Smith et al., 1990; Harrison et al., 1992; Vasilikiotis and Melis, 1994). In the following, sample loadings for SDS-PAGE in LL- and HL-grown cells will be given on a per Chl basis. However, the interested reader can readily convert results to a per cell basis.

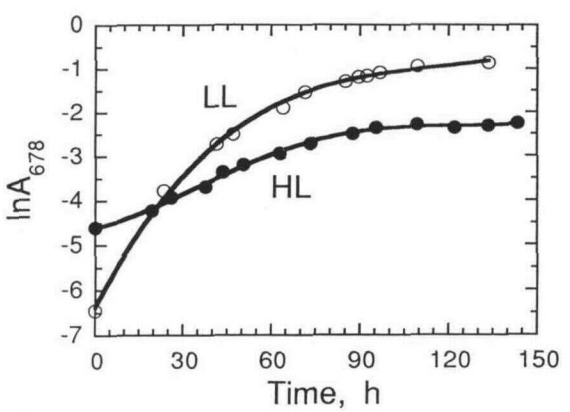

Figure 1. Growth curve of *D. salina* under LL and HL conditions. The ln A_{678} is plotted as a function of time during cell growth. LL cells (○) were grown under 100 μmol photons m^{-2} s^{-1}. HL cells (●) were grown under 2000 μmol photons m^{-2} s^{-1}.

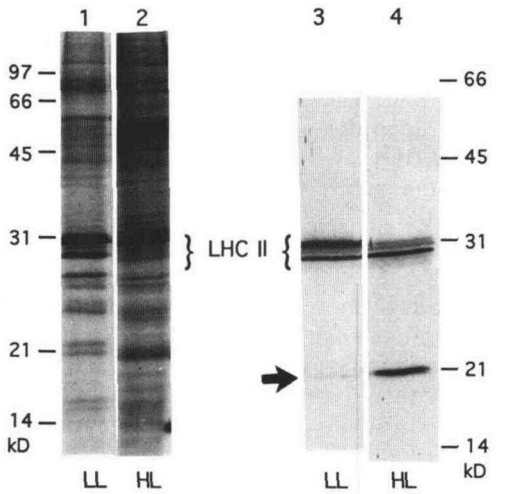

Figure 2. SDS-PAGE and western blot analysis of polypeptides from LL- and HL-grown *D. salina* thylakoid membranes. Lanes 1 and 2 are Coomassie-stained gels showing the polypeptide profile of LL thylakoids (lane 1) loaded with 6 nmol of Chl and HL thylakoids (lane 2) loaded with 3 nmol of Chl. Note the overall greater amount of protein in the HL versus LL thylakoids and the distinct depletion of protein bands associated with the LHC-II in the HL thylakoids. Lanes 3 and 4 show western blots probed with polyclonal antibodies against the LHC-II. Cross-reactivity of the LHC-II antibody was significantly lower in the HL (lane 4) than in the LL thylakoids (lane 3). Note also the cross-reaction between the polyclonal antibodies and a low molecular mass polypeptide (approximately 20 kD, arrow), predominant in the HL sample only (lane 4).

SDS-PAGE and Immunoblot Analysis of Thylakoid Membrane Polypeptides from LL- and HL-Grown *D. salina*

The response of the photosynthetic apparatus in *D. salina* to irradiance stress involves many interrelated phenomena, one of which is the down-regulation of the Chl antenna size of the LHC-II. Figure 2 presents a comparison of the SDS-PAGE polypeptide profile of *D. salina* thylakoids grown under LL and HL conditions. Lane 1 shows thylakoid proteins (6 nmol of Chl loaded) from an LL-grown sample. The proteins of the LHC-II appear as a set of dominant, strongly staining bands in the 28- to 31-kD region of the gel and clearly are the most abundant proteins of the thylakoid membrane. In contrast, the LHC-II proteins of the HL-grown sample (lane 2, 3 nmol of Chl loaded) are greatly diminished relative to other membrane proteins. That the relative abundance of the LHC-II decreases in HL-grown thylakoids is further evidenced in immunoblots probed with polyclonal antibodies against the apoproteins of the LHC-II. Figure 2 (lane 3) shows the cross-reaction between such polyclonal antibodies and LHC-II proteins from LL-

grown *D. salina* (equivalent of 6 nmol of Chl). Figure 2 (lane 4) shows the cross-reaction between the polyclonal antibodies and proteins from HL-grown *D. salina* (equivalent of 3 nmol of Chl loaded). It is evident that considerably fewer LHC-II apoproteins are associated with the thylakoid membrane of HL-grown *D. salina*. This observation is consistent with the results of other investigators (Sukenik et al., 1988; Smith et al., 1990) and suggests that one adaptive response of green algae to high irradiance stress is the down-sizing of the Chl *a-b* LHC-II antenna by an as-yet unknown mechanism.

The western blot analysis (Fig. 2, lanes 3 and 4) revealed that the more abundant higher molecular mass proteins of the LHC-II (approximately 31 kD) were selectively depleted from the thylakoid membrane under HL growth conditions. Conversely, an LHC-II protein at approximately 28 kD was preferentially retained in the thylakoid membrane of HL-grown *D. salina*. These results suggest that the level of irradiance during cell growth exerts a differential regulation on the expression of *Lhcb* genes and/or on the assembly of the LHC-II polypeptides in the Chl *a-b* light-harvesting antenna. A more detailed analysis on this differential regulation of LHC-II apoproteins by irradiance is presented below.

Of interest in the results of Figure 2 (lane 4, HL sample) is the cross-reaction of the LHC-II polyclonal antibodies with an approximately 20-kD polypeptide (marked by arrow). Such a cross-reaction is much less pronounced, or absent, with proteins from the LL sample (lane 3). This novel protein (approximately 20-kD protein) was consistently present in the samples isolated from the HL-grown cells. Occasionally, a second cross-reaction was detected with a band in the approximately 18-kD region (not shown here).

To explore the kinetics of the differential expression of *Lhcb* genes and of the appearance of the approximately 20-kD protein, we conducted light-shift experiments in which LL-grown algae were switched to HL conditions and vice versa.

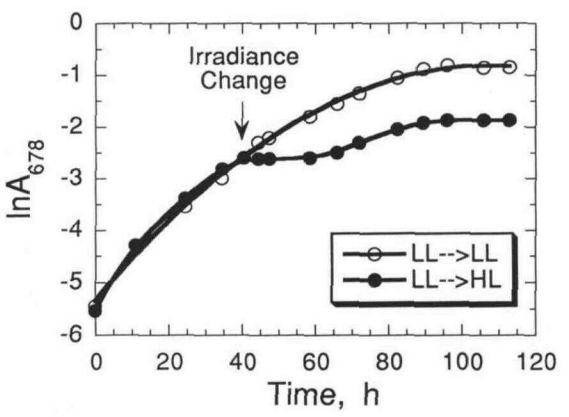

Figure 3. Effect of irradiance change from LL to HL on Chl accumulation and growth of *D. salina*. Cultures were grown under LL conditions at 100 μmol photons m^{-2} s^{-1} and either maintained at this irradiance (○) or switched to HL conditions (2000 μmol photons m^{-2} s^{-1}) at 40 h (●). Cell growth was measured as the ln A_{678}.

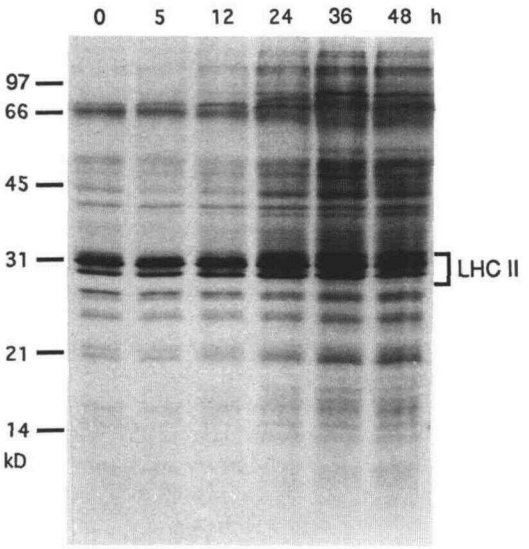

Figure 4. SDS-PAGE profile of solubilized membrane proteins from thylakoids of *D. salina* transferred from LL to HL growth conditions as shown in Figure 3. Cells were harvested at the time of transition (0 h) and at subsequent time intervals as indicated. Thylakoid membranes from these cells were isolated, solubilized, and loaded at 3.8 nmol of Chl per lane.

Effect of Irradiance Change (LL→HL shift) on *D. salina* Thylakoid Membrane Protein Profile

When LL-grown cultures, still in the linear logarithmic phase of growth, are switched to HL conditions they gradually lose their deep green color and acquire the greenish-yellow appearance of HL cells. Figure 3 shows the growth curve of an LL culture of *D. salina* (open circles) and that of a replicate LL culture that was switched to HL conditions in the mid-logarithmic phase of growth (solid circles, 40 h, ln A_{678} = −2.6). It can be seen that the LL control culture continues to accumulate Chl and reaches the stationary phase approximately 100 h later at ln A_{678} = −0.9. The LL→HL culture shows a lag of about 20 h in Chl accumulation after the irradiance change. Subsequently, Chl accumulation resumes at a slower rate than that of the LL control. The LL→HL culture reaches the stationary phase at a lower density ln A_{678} = −2.0, which is typical for HL-grown cells (Fig. 1). We have determined that during this transition period, cell division continues, albeit at a slower rate. The results show that the rate of Chl biosynthesis is lowered promptly upon transfer to HL conditions. Moreover, these results underline changes in the amount and organization of the LHC-II in chloroplasts, occurring upon the LL→HL transition (see below).

Thylakoid membrane proteins from cells that had been transferred from LL to HL conditions were examined by SDS-PAGE. In this experiment, an LL culture of *D. salina* was transferred to HL conditions, cells were harvested after different periods of exposure to HL, thylakoids were isolated and solubilized, and proteins were resolved based on equal Chl loading (3.8 nmol per lane) as described previously. In Figure 4, a Coomassie-stained gel from such an experiment shows that the amount of total protein per

lane increases but the relative amount of LHC-II protein decreases with time of exposure to HL, a consequence of the decrease in the Chl/protein ratio occurring as the Chl antenna of PSII is down-sized under the HL conditions.

Figure 5 shows a western blot analysis of such samples using polyclonal antibodies to the LHC-II. It can be seen that gradual changes occur in the relative subunit composition of the LHC-II as a function of time in HL. Higher molecular mass subunits of the LHC-II (the approximately 31-kD region) specifically decrease in abundance relative to the lower molecular mass subunit at approximately 28 kD. This probably reflects a shift in the relative subunit composition of the LHC-II, occurring concomitantly with the adjustment in the LHC-II antenna size.

Important in the results of Figure 5 is the appearance of a protein band in the approximately 20-kD region that cross-reacts with the anti-LHC-II antibodies. This protein (Fig. 5, arrow) accumulates steadily as a function of time in HL from nondetectable levels in LL samples (0 h) to levels after 48 h of HL exposure that are sufficient to give a cross-reaction with the LHC-II antibody comparable in intensity with that of the LHC-II subunits. It appears that the transition to HL causes either the de novo biosynthesis of an approximately 20-kD protein or its generation from existing proteins in the thylakoid membrane, or both.

Figure 6 is the result of densitometric scans of western blots similar to those shown in Figure 5. The results show a 2-fold increase in the relative amount of the smaller LHC-II subunits (Fig. 6, 28 kD) and a corresponding decline in the amount of the larger subunits. As a result, the relative amount of the total LHC-II in the thylakoid membrane remained fairly constant during the 48-h period in HL (Fig. 6, LHC-II). Note also the kinetics of the approximately 20-kD protein increase as a function of culture time in HL.

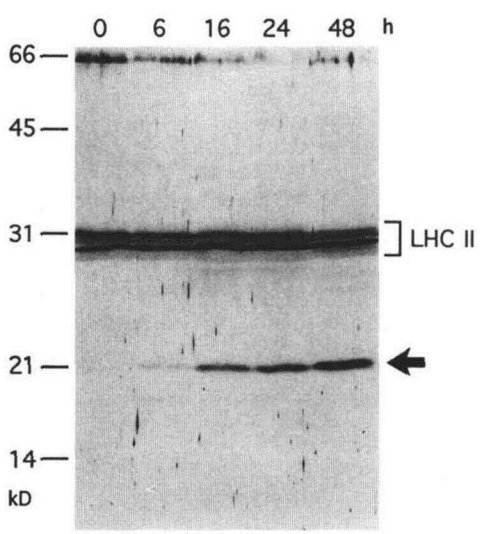

Figure 5. Western blot analysis of thylakoid membrane proteins from LL-grown *D. salina* cells after transition to HL. Cells were grown under LL conditions and then transferred to HL conditions as shown in Figure 3. Cells were harvested at zero time after the light shift and at subsequent time intervals as indicated. Thylakoid membranes were isolated and analyzed by SDS-PAGE as described in Figure 4. Polyclonal antibodies against the LHC-II were used to obtain the immunoblot shown.

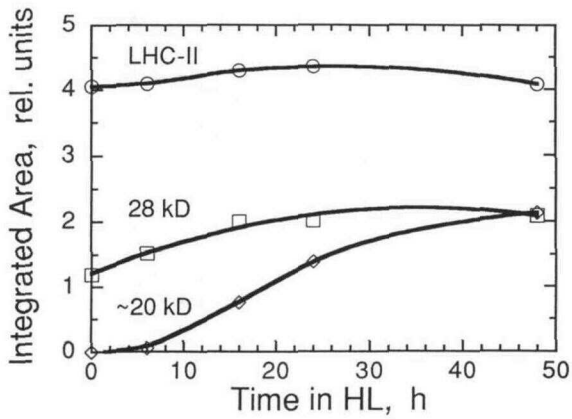

Figure 6. Quantitation of cross-reactions following a transition of *D. salina* cells from an LL to an HL environment. The relative amount of the total LHC-II (○), the lower (28 kD) molecular mass subunits (□), and the approximately 20-kD proteins (◇) were measured from the densitometric analysis of western blots similar to that shown in Figure 5.

Effect of Irradiance Change (HL→LL shift) on *D. salina* Thylakoid Membrane Protein Profile

In the converse experiment, in which an HL-grown culture was transferred to LL conditions, the greenish-yellow typical of the HL cultures changed to deep green within hours of exposure to LL conditions. Figure 7 shows the growth curve of an HL-grown culture (open circles) and an HL culture transferred to LL conditions at 40 h and an ln $A_{678} = -3.5$. Here, the HL culture reached maximal density at ln $A_{678} = -1.9$, whereas the HL→LL culture reached a maximum ln A_{678} value of -1.0. We observed that, upon transition to LL conditions, enhanced accumulation of Chl in the culture is prompt and approaches a rate comparable to that of the rate encountered in LL-grown cultures.

Thylakoid membrane proteins from cells that had been transferred from HL to LL conditions were examined by SDS-PAGE. Cells were harvested after different periods of exposure to LL, membranes were isolated and solubilized, and proteins were resolved based on equal Chl loading (4 nmol per lane). Figure 8 shows a Coomassie-stained gel from such an experiment in which it can be seen that the amount of total protein per lane decreases with increasing time of exposure to LL, a consequence of the increase in the Chl/protein ratio expected as the Chl antenna size increases under the LL conditions. Moreover, there is a gradual increase in the amount of the LHC-II present, as well as changes in the relative subunit composition of the LHC-II as a function of time in LL.

Figure 9 shows a western blot analysis of such samples using polyclonal antibodies raised against the LHC-II. It can be seen that during a 48-h period in LL there is a gradual change in the amount and subunit composition of the LHC-II. At 0 h, the LHC-II of the HL-grown cells

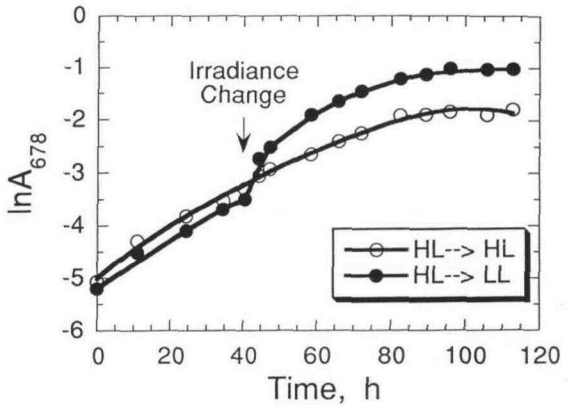

Figure 7. Effect of irradiance change from HL to LL on the growth of *D. salina*. Cultures were grown under HL conditions (2000 μmol photons m^{-2} s^{-1}) and either maintained at this irradiance (○) or switched to LL conditions (100 μmol photons m^{-2} s^{-1}) at 40 h (●). Cell growth was measured as the ln A_{678}.

appears to contain only two subunits migrating at approximately 28 and 28.5 kD. After 2 h in LL, a higher molecular mass subunit of approximately 31 kD is clearly present. This is followed after 6 h by the appearance of minor amounts of a lower molecular mass subunit at approximately 27 kD. With increasing time in LL the relative intensity of the LHC-II subunits is seen to increase. The rate of increase, however, is faster for the approximately 31-kD and slower for the 28- and 28.5-kD subunits.

Figure 9 also shows that, in a complementary fashion to the LL→HL experiment, the amount of the approximately 20-kD protein decreases gradually over time in LL. The sample from the HL-grown culture (0 h lane) contains significant levels of the approximately 20-kD protein, giving a cross-reaction with the polyclonal LHC-II antibodies

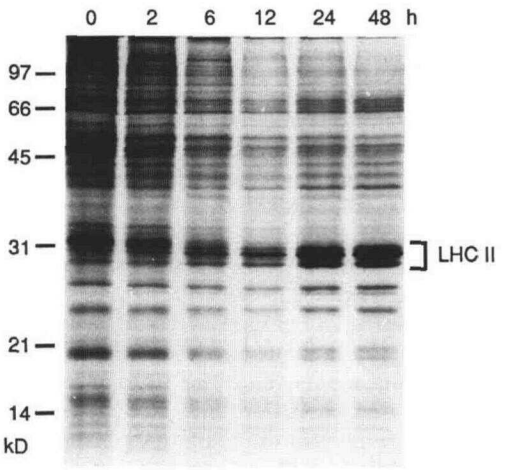

Figure 8. SDS-PAGE profile of solubilized membrane proteins from thylakoids of *D. salina* transferred from HL to LL as shown in Figure 6. Cells were harvested at zero time after the light shift (0 h) and at subsequent time intervals as indicated. Thylakoid membranes from these cells were isolated, solubilized, and loaded at 4 nmol of Chl per lane.

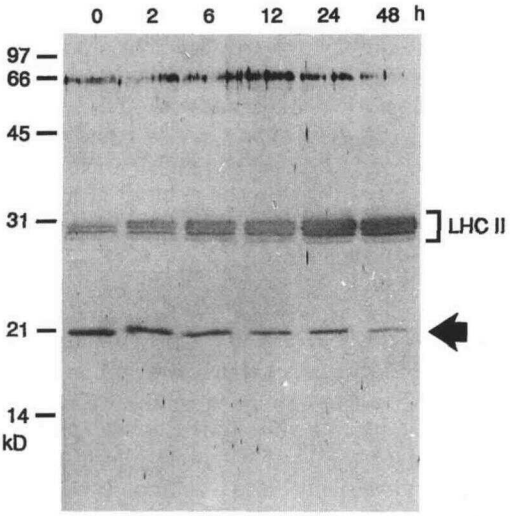

Figure 9. Western blot analysis of thylakoid membrane proteins from HL-grown *D. salina* cells after transition to LL. Cells were grown under HL conditions and then transferred to LL conditions as shown in Figure 7. Following the light shift, cells were harvested at the time intervals indicated and thylakoid membranes were isolated and analyzed by SDS-PAGE as described in Figure 8. Polyclonal antibodies to the LHC-II were used to obtain the immunoblot shown. The cross-reacting proteins visible in the 27- to 31-kD region are those of the LHC-II. The approximately 20-kD protein appears as a cross-reacting band (arrow) and clearly decreases in abundance with increasing time of exposure to LL.

that is considerably more intense than that of the LHC-II itself. During time of exposure to LL, however, this relationship gradually becomes reversed as the relative level of the approximately 20-kD protein decreases significantly and the level of LHC-II increases.

Figure 10 presents the result of densitometric scans of western blots similar to those shown in Figure 9. The results show a 2-fold increase in the relative amount of the smaller LHC-II subunits during the 48-h period in HL (Fig. 10, 28 kD). However, there is 4-fold increase in the amount of the total LHC-II in the thylakoid membrane (Fig. 10, LHC-II), reflecting a significantly greater increase in the amount of the higher molecular mass proteins of the LHC-II (approximately 31 kD). Note also the kinetics of the decrease in the approximately 20-kD protein as a function of cell time in LL.

DISCUSSION

Both Chl *a* and Chl *b* are used by higher plants and green algae as the main light-harvesting pigments; however, these pigments are not homogeneously distributed among the various Chl-binding proteins. Since the majority of Chl *b* is associated with the LHC-II, the Chl *a*/Chl *b* ratio provides information concerning the size of the LHC-II that stably assembles relative to the Chl *a*-binding proteins of the PSII core. Evidence in the literature (Anderson, 1986; Melis, 1991) suggests that variations in the Chl *a*/Chl *b* ratio occur naturally in higher plants and green algae in response to long-term changes in irradiance. This response

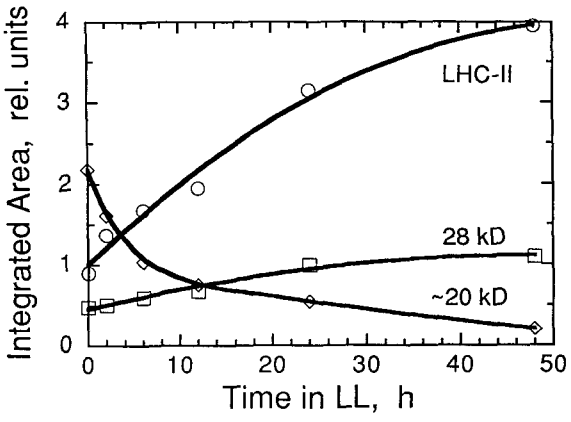

Figure 10. Quantitation of cross-reactions following a transition of *D. salina* cells from an HL to an LL environment. The relative amount of the total LHC-II (○), the lower molecular mass subunits of the LHC-II (28 kD; □), and the approximately 20-kD proteins (◇) were measured from the densitometric analysis of western blots similar to that shown in Figure 9.

appears to be well conserved in all photosynthetic systems examined. For example, LL-grown *D. salina* has a Chl *a*/Chl *b* ratio in the range of 3 to 5 and an LHC-II containing approximately 250 Chl (*a* plus *b*) molecules per PSII complex. HL-grown *D. salina* has a Chl *a*/Chl *b* ratio greater than 10 and an LHC-II containing approximately 30 Chl (*a* plus *b*) molecules per PSII complex (Smith et al., 1990). Similar conclusions can be inferred from the results reported for the green alga *Chlorella vulgaris* (Ley and Mauzerall, 1982). These observations show that one adaptive response of green algae to high irradiance is the downsizing of the LHC-II antenna system by an as yet unknown mechanism.

This investigation has shown that acclimation of *D. salina* to the level of irradiance involves distinct changes in the abundance of protein associated with the LHC-II and in the polypeptide composition of this complex. The kinetics of change suggest different mechanisms for the response of LL-acclimated cells to HL versus the response of HL-acclimated cells to LL.

In LL-acclimated cells, thylakoid membranes contain a fully assembled LHC-II complex. A transition to HL does not bring about gross and prompt changes in the amount of the total LHC-II. Rather, gradual changes in the amount and relative LHC-II subunit composition take place with an increase in the relative amount of the smaller (approximately 28 kD) subunits and a corresponding decrease in the relative amount of the larger (approximately 31 kD) subunits (Fig. 5). We estimated that the kinetics of the increase in the approximately 28 kD subunit (Fig. 6) parallel those of the increase in the cell density of the culture as a function of time in HL. This analysis suggests that, following an LL→HL transition, the LHC-II of newly synthesized PSII units preferentially contain the approximately 28-kD subunits and lack the approximately 31-kD subunits. Thus, the adjustment of the LHC-II antenna size in this case involves a fine regulation in the biosynthesis and assembly of new LHC-II subunits. The acclimation of a culture to the HL condition, therefore, comes about by cell growth and by "dilution" in the thylakoid membrane of fully assembled LHC-IIs. It does not appear to involve a wholesale and immediate degradation of pre-existing LHC-IIs.

In HL-acclimated cells, thylakoid membranes contain PSII complexes with a highly truncated LHC-II. Upon transfer of HL-acclimated cells to LL conditions, we observed a prompt decrease in the Chl *a*/Chl *b* ratio (not shown) and a concomitant increase in the Chl antenna size of the photosystems. We estimated that the kinetics of the increase of the approximately 28-kD subunit in this experiment (Fig. 10) lagged significantly behind the increase of Chl in the culture, observed as a function of time in LL (Fig. 7). However, under these experimental conditions, the kinetics of the total LHC-II increase were 200% faster than those of the approximately 28-kD subunit (Fig. 10), underlining an even faster rate of biosynthesis for the approximately 31-kD subunits. This analysis suggests that the approximately 31-kD subunits are utilized as peripheral components of the LHC-II both in the newly synthesized PSII complexes and as an addition to those PSII units that were assembled earlier with a truncated LHC-II under the HL growth conditions.

In conjunction with the above observations, we noted the appearance of a novel protein having a molecular mass of approximately 20 kD that appears to accumulate in *D. salina* chloroplasts under irradiance-stress conditions. This novel protein must contain epitopic regions with relatively high homology to regions on proteins of the LHC-II because it cross-reacts with specific polyclonal antibodies raised against the LHC-II.

The approximately 20-kD protein may represent a degradation product of the LHC-II, originating from the proteolysis of de novo synthesized LHC-II polypeptides, which were imported into the chloroplast of *D. salina* but failed to stably assemble in the thylakoid membrane for lack of sufficient Chl under HL growth conditions. This possible mechanism has merit because it is known that the Chl antenna size of the photosystems and the stability of LHC-II apoproteins are both regulated at the holocomplex assembly level by the availability of Chl *b* (Bellemare et al., 1982; Peter and Thornber, 1991). This scenario is consistent with results in Chl *b*-less mutants in which the LHC-II apoproteins are synthesized but do not accumulate in the thylakoids (Bellemare et al., 1982; Terao and Katoh, 1989). It was implied that the apoproteins are unstable and are degraded even though a small amount may still be inserted into the thylakoid membrane without Chl. It has also been shown (Reed et al., 1990; Honda et al., 1991) that under Chl deficiency conditions, the proteins of the LHC-II appear to be proteolytically degraded to fragments migrating in the 15- to 20-kD region.

Alternative explanations for the origin of the novel approximately 20-kD protein do exist, however. It is possible that the approximately 20-kD protein is a nuclear- or plastid-encoded polypeptide that is homologous to the LHC-II polypeptides (homology sufficient to yield a cross-reaction with the LHC-II antibodies). This polypeptide could be induced under HL stress conditions and may play a sig-

nificant functional role in the stressed chloroplast. Both ELIP-type proteins and the *Cbr* gene product exhibit a high degree of homology to the proteins of the LHC and appear to play unique roles in the chloroplast defense against light-induced stress (Green and Pichersky, 1994).

ELIPs are nuclear-encoded membrane proteins that occur transiently in the early stages of the light-induced development of chloroplasts from etioplasts (Grimm et al., 1989) and in mature plants in response to light stress (Adamska et al., 1993). Once integrated into the thylakoid membrane, ELIPs appear to associate with PSII, although they have not been shown to bind Chl. It has been proposed that, in the early stages of photosystem assembly (etioplast greening), they act as temporary substitutes for the LHC proteins, which do not accumulate to significant levels until after the appearance of the ELIPs (Adamska and Kloppstech, 1991). The appearance of stable, membrane-associated ELIPs in mature plants under light-stress conditions or in the presence of carotene synthesis inhibitors suggests that they may play a role in photoprotection of PSII (Adamska et al., 1993).

An interesting, and possibly related, response of the green alga *D. bardawil* to HL stress is the accumulation of high levels of β-carotene, presumably as a protective measure against photooxidative damage. The nuclear gene designated *Cbr* was isolated from an HL-induced cDNA expression library from *D. bardawil* (Levy et al., 1992). The fact that *Cbr* transcription and β-carotene biosynthesis show coincidental modes of induction and that certain nucleotide sequences upstream of *Cbr* match consensus sequences of the sterol-response elements coding for mevalonic acid biosynthesis suggested that *Cbr* is co-regulated with genes crucial for β-carotene biosynthesis. The predicted amino acid sequence of *Cbr* shows a high degree of homology to the ELIP proteins and, to a lesser degree, to the proteins of the LHC. Analysis by nondenaturing PAGE indicated that Cbr proteins are integrated into the thylakoid membrane and exist in association with the LHC-II. It seems likely, based on these facts, that Cbr proteins are also involved in photoprotection. More recently, evidence was presented that shows that the Cbr protein specifically binds zeaxanthin, further supporting a possible anti-photooxidative role for this protein (Levy et al., 1993). Interestingly, antibodies raised against the predicted *Cbr* gene product have been shown to cross-react strongly with a thylakoid membrane protein from the chloroplasts of nutrient-stressed *D. salina*, even though the latter does not accumulate β-carotene to significant levels under such stress.

The gene product of the nuclear gene *PsbS*, which has been reported in higher plants and cyanobacteria, is known to be a 22-kD protein and a member of the LHC superfamily (Green and Pichersky, 1994). Unlike the other members of this family of proteins, the *PsbS* protein contains an extra transmembrane helix in addition to the normal three but still includes large regions of highly conserved amino acid sequences. It too appears to be associated closely with the PSII and binds both Chl *a* and Chl *b* (Funk et al., 1994), but it has not been shown to be involved in a stress response.

Other 20- to 22-kD chloroplast-localized proteins are induced under stress (particularly heat stress) conditions. The so-called low-mol-wt heat-shock proteins are members of another large superfamily of proteins shown to occur in higher plants in response to heat-stress conditions. A chloroplastic heat-shock protein of approximately 21-kD (Vierling, 1991) has been well characterized in several plant species and, although this group of proteins appears to bear no structural similarity to the LHC family of proteins, their induction and localization in the chloroplast under conditions similar to those described here bears noting. A further detailed characterization of the novel approximately 20-kD protein is currently underway in our laboratory.

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