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Elimination of high-light-inducible polypeptides related to eukaryotic chlorophyll *a*/*b*-binding proteins results in aberrant photoacclimation in *Synechocystis* PCC6803

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Abstract

The *hli* genes, present in cyanobacteria, algae and vascular plants, encode small proteins [high-light-inducible polypeptides (HLIPs)] with a single membrane-spanning α-helix related to the first and third helices of eukaryotic chlorophyll *a*/*b*-binding proteins. The HLIPs are present in low amounts in low light and they accumulate transiently at high light intensities. We are investigating the function of those polypeptides in a *Synechocystis* PCC6803 mutant lacking four of the five *hli* genes. Growth of the quadruple *hli* mutant was adversely affected by high light intensities. The most striking effect of the quadruple *hli* mutation was an alteration of cell pigmentation. Pigment changes associated with cell acclimation to increasing light intensity [i.e. decrease in light-harvesting pigments, accumulation of the carotenoid myxoxanthophyll and decrease in photosystem I (PSI)-associated chlorophylls] were strongly exacerbated in the quadruple *hli* mutant, resulting in yellowish cultures that bleached in high light and died as light intensities exceeded (>500 μmol photon m^{-2} s^{-1}). However, these pigment changes were not associated with an inhibition of photosynthesis, as probed by in vivo chlorophyll fluorescence, photoacoustic and O_2-evolution measurements. On the contrary, the HLIP deficiency was accompanied by a stimulation of the photochemical activity, especially in high-light-grown cells. Western blot analyses revealed that the PSI reaction center level (PsaA/B) was noticeably reduced in the quadruple *hli* mutant relative to the wild type, whereas the abundance of the PSII reaction center protein D1 was comparatively little affected. The *hli* mutations did not enhance photoinhibition and photooxidation when cells were exposed over a short term to a very high light intensity. Together, the results of this study indicate that HLIPs are critical in the adaptation of the cyanobacterium to variations in light intensity. The data are consistent with the idea that HLIPs are involved, through a direct or indirect means, in nonphotochemical dissipation of absorbed light energy.

Keywords: Chlorophyll; Light acclimation; Nonphotochemical energy dissipation; Photosynthetic electron transport; Xanthophyll; *Synechocystis* PCC6803

1. Introduction

Oxygenic photosynthesis of plants and cyanobacteria involves the tandem operation of photosystems II and I (PSII and PSI). Each photosystem is composed of two substructures: a reaction center that generates a light-induced charge separation and an antenna system that absorbs excitation energy and transfers it to the photochemical reaction center complex. While the reaction centers are remarkably conserved throughout evolution, antenna pigment–protein complexes of cyanobacteria differ markedly from those of vascular plants or green algae. In the latter organisms, photons are collected by chlorophyll *a*/*b*–protein complexes (LHCs) that are encoded by a multigenic family [1–3]. According to the high-resolution structure of the major Lhc polypeptide of PSII (LHCII), each Lhc polypeptide binds seven chlorophyll *a*, five chlorophyll *b*, two lutein molecules and a nonstoichiometric amount of violaxanthin and neoxanthin [4]. The extended Lhc protein family also includes the early-light-inducible proteins (ELIPs), which are homologous to the Lhc polypeptides

Abbreviations: ELIP, early-light-inducible protein; HLIP, high-light-inducible polypeptide; MDA, malondialdehyde; NPQ, nonphotochemical energy quenching; PFD, photon flux density; PS, photosystem; WT, wild type

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but differ from them by their transient accumulation during chloroplast greening and during exposure of plants to high light stress [5]. Biochemical analyses of purified ELIPs have shown that these stress-induced proteins bind both chlorophyll and xanthophyll [6]. While most members of the Lhc family have three membrane-spanning helices, there are a number of notable exceptions. The PSII-S protein has four membrane-spanning regions [7] while genes encoding small Lhc-type polypeptides with one or two putative transmembrane helices are encoded on the *Arabidopsis* genome [8,9]. Those small Lhc-like proteins, believed to be ancestral to ELIPs and Lhc polypeptides, appear to accumulate in response to the absorption of excess excitation energy.

In contrast to vascular plants, cyanobacteria lack the multi-helix Lhc polypeptides. The major peripheral light-harvesting complex of many cyanobacteria is the water-soluble phycobilisome which contains phycobilin pigments that are covalently bound to the antenna polypeptides [10]. However, it was recently discovered that cyanobacteria possess single-helix polypeptides related to Lhc proteins; these proteins have been designated 'high-light-inducible proteins' (HLIPs) or 'small Cab-like proteins' (SCPs). The genes encoding the HLIPs, or *hli* genes, were first identified in *Synechococcus* sp. strain PCC7942 [11] and subsequently found in other cyanobacteria [12], in red algae [13] and more recently, in the vascular plant *Arabidopsis thaliana* [8]. In *Synechocystis* PCC6803, there are four monocistronic *hli* genes that compose an *hli* multigene family [14]. In addition to these four small genes (denoted *hli*A-D), there is a fifth gene (*hli*E) encoding ferrochelatase, an enzyme of the porphyrin biosynthesis pathway, that is fused at the C terminus to an HLIP domain.

Expression of the *hli* genes in both cyanobacteria and vascular plants is similar to that of the ELIP genes, that is, the *hli* mRNAs and encoded proteins accumulate under various conditions that result in excess absorbed light energy (relative to what can be dissipated through the photosynthetic processes), including high light intensity, nitrogen starvation or low temperature [8,11,15]. Using cell lines in which the *hli* polypeptides were tagged with a His_6 epitope, He et al. [15] showed that HliA and HliC accumulated more than HliB and HliD in *Synechocystis* PCC6803 exposed to high light. Also, these authors observed that the accumulation of the different *hli* polypeptides exhibited specificity with respect to a diverse range of environmental conditions. However, low levels of HLIPs were also observed in *Synechocystis* cells grown under light conditions that did not cause apparent light stress [14,15].

It is not yet known whether the HLIPs bind pigments. Single mutant *Synechocystis* strains affected in one of four *hli* genes were not particularly susceptible to high light [14,15], although some of the single mutants could not compete with wild-type (WT) cells under high-light conditions [15]; WT cells overgrow the mutant strain during co-cultivation under high-light conditions. A strain deleted for all four of the *Synechocystis hli* genes was shown to lose variable chlorophyll fluorescence when exposed to very high light [15]. The molecular bases of photosensitivity of the mutant strain and the ways in which the HLIPs might function in protection are unknown. As the mutant and WT strains were equally sensitive to methylviologen [15], which catalyzes superoxide formation in the light, the quadruple mutant is not likely to be defective for activities involved in detoxification of reactive oxygen species. By analogy with vascular-plant ELIPs, a number of functions have been suggested for HLIPs. They may associate with pigments, perhaps transiently, serving as chlorophyll carriers [14] and function to regulate tetrapyrrole biosynthesis [16], or they may function in the dissipation of excess absorbed light energy within antennae complexes [8,17]. The fusion of the fifth HLIP with ferrochelatase could be important for protection against the potentially toxic effects of photoactive porphyrin intermediates [8,14].

In this paper, we perform a detailed study of the light responses of a quadruple *hli* mutant of *Synechocystis* PCC6803 which lacks all four of the small HLIPs (HliA, HliB, HliC and HliD). The results confirm the importance of HLIPs for photoacclimation of *Synechocystis*. Loss of the HLIPs strongly modified cell pigmentation and altered the extent to which the cyanobacterium performs non-photochemical dissipation of absorbed light energy. The involvement of HLIPs in energy dissipation may be direct, suggesting a function partly analogous to that of the nonphotochemical energy quenching (NPQ) process used by vascular plants to regulate the utilization of excitation energy by the photosynthetic apparatus, or indirect, through organisational changes of the photosynthetic apparatus, for example, via regulation of chlorophyll synthesis [16].

2. Materials and methods

2.1. Strains and growth conditions

Synechocystis PCC6803 was grown in BG-11 inorganic medium buffered with 10 mM TES (pH 8.2), at 30 °C [18]. A *Synechocystis* PCC6803 strain in which the four *hli* genes encoding the small HLIPs were inactivated by insertion of a drug-resistant marker gene [15] was used. This quadruple *hli* mutant was grown in medium supplemented with kanamycin, spectinomycin, chloramphenicol and erythromycin (25 μg/ml for each antibiotic). Cultures were gently stirred and illuminated with continuous white light produced by neon tubes (Sylvania standard cool white F58W/133-T8). Photon flux densities (PFDs) were measured with a Li-Cor quantum-meter (LI-185B/LI-190SB). Cell density was estimated by measuring the optical density (OD) of the suspension at 730 nm (OD_{730}) with a spectrophotometer (Uvikon 942, Kontron). Cells were diluted to an OD_{730} of 0.2 with precultures grown in low light (10 μmol photon

m^{-2} s^{-1}) before transfer to new light conditions (either high light or low light). Cells were grown as 50 ml cultures in 250 ml Erlenmeyer flasks.

Cell density was also determined by counting *Synechocystis* cells with a Malassez cell. The relationship between OD_{730} and cell number was identical for WT and mutant cell suspensions grown in low light (10 μmol photon m^{-2} s^{-1}) or in high light (100 μmol photon m^{-2} s^{-1}). An OD_{730} of 1 corresponded, on average, to 9.0 ± 0.7 and $8.8 \pm 0.2 \times 10^7$ cells ml^{-1} for WT and mutant cells grown in low light and to 8.5 ± 0.3 and $8.6 \pm 0.1 \times 10^7$ cells ml^{-1} for WT and mutant cells grown in high light, respectively.

2.2. Pigment analyses

Cell suspensions were frozen in liquid nitrogen before analysis. After thawing, cells were pelleted by centrifugation and pigments extracted in pure methanol, at 4 °C and in dim light. After centrifugation and filtration, pigments were separated by high-performance liquid chromatography (HPLC) on a reverse phase C18 column (Waters Nova Pak, 60 A, 4 μm, 3.0×300 mm) protected by a Bondapak C18 guard column, using the method developed by Lagarde et al. [19]. Pure pigments used for calibration were bought from Extrasynthèse (Genay, France) or were prepared by TLC with *n*-hexane/isopropanol (100:10; v/v) as solvent system.

2.3. Western blot analyses

Cell suspensions were centrifuged and resuspended in 50 mM Tris (pH 8), 0.6 M sucrose, 1 mM phenylmethylsulfonyl fluoride and 1 mM aminobenzamidine. Cells were disrupted by three successive passages in a French press. After centrifugation at low speed (5 min, 5000 rpm) to remove unbroken cells and cellular debris, the supernatant was centrifuged for 1 h at $100{,}000 \times g$. Pelleted membranes were suspended in 50 mM Tris (pH 8) and solubilized by adding half volume of solubilization buffer [6% SDS, 5 mM EDTA, 75 mM Tris (pH 6.8), 20% glycerol, 200 mM DTT] and by incubation at 45 °C for 20 min. After centrifugation, 15 μg/lane membrane proteins were separated by SDS-PAGE in a 13% polyacrylamide gel containing 6 M urea, according to Laemmli [20]. Concentration of thylakoid membrane polypeptides was determined using a modified Lowry method using bovine serum albumin as a standard (Sigma). Proteins were electrophoretically transferred to nitrocellulose membranes and immunodetection of D1 and PsaA was performed with *Synechocystis* anti-D1 and anti-PsaA antibodies. Bound antibodies were detected using anti-rabbit IgG conjugated to alkaline phosphatase (Boehringer). The antibodies used in this study were kindly provided by Dr. E.B. Gonzalez (University of Turku, Finland) and Dr. H.B. Pakrasi (Washington University, St. Louis, MO).

2.4. O_2 photoproduction measurements

O_2 exchange by cell suspensions ($OD_{730} = 0.8$) supplemented with bicarbonate was measured at 30 °C with a Clark-type O_2 electrode (Hansatech DW2/2). Cells were illuminated with white light produced by a Schott KL1500 lamp equipped with a flexible light guide. PFDs were adjusted with Schott neutral density filters and were measured in the cuvette with the 2060-M light sensor of a PAM-2000 fluorometer (Walz).

2.5. Chlorophyll fluorescence measurements

Room-temperature chlorophyll fluorescence was measured with a PAM-2000 modulated fluorometer (Walz), as previously described [21]. The minimal level of chlorophyll fluorescence (F_o) was generated by a weak red light modulated at 600 kHz and was determined after a brief illumination (2 s) with far-red light. The maximal level of chlorophyll fluorescence (F_m) was generated by an 800-ms flash of intense white light. From the F_o and F_m values, the maximal quantum yield of PSII photochemistry was determined as $(F_m - F_o)/F_m = F_v/F_m$. The F_v/F_m measurements were performed after dark adaptation or after cells were placed into state 1 by illumination with white light in the presence of DCMU, as described in Ref. [22]. The actual quantum yield of PSII photochemistry was determined in illuminated cells from the maximal fluorescence (F'_m) and the steady-state level (F_s) as $(F'_m - F_s)/F'_m$. The PSII quantum yield was also measured with the PSII electron acceptor, 2,5-dichloro-1,4-benzoquinone (DCBQ) [23]. $K_3Fe(CN)_6$ (3.75 mM) and DCBQ (300 μM) were added sequentially to the cell suspensions 3 min before illumination. Other PSII electron acceptors were also used: 600 μM diaminodiurene (DAD) [+3.75 mM $K_3Fe(CN)_6$] and 150–200 μM 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB).

2.6. Photoacoustic measurements

Cyanobacterial cells deposited on a nitrocellulose filter (Millipore SCWP01300, 8 μm) were placed in the hermetically closed cell of a photoacoustic spectrometer that has been described [24]. Light from an Oriel 1000-W halogen lamp was filtered through a broadband interference red or green filter (Oriel 57610 filter with a peak wavelength of 645 nm or Oriel 57550 filter with peak wavelength of 510 nm) and was chopped at 11 Hz with a mechanical chopper. The PFD of the resulting red or green light was 20 or 12 μmol photon m^{-2} s^{-1}, respectively. The signal recorded by the microphone was fed into a two-phase lock-in amplifier and was analyzed as previously described [24 (see also Ref. [25]). Photochemistry was saturated with strong white background light (1500 μmol photon m^{-2} s^{-1}), leading to maximal heat emission. From the amplitude of this light-saturated photothermal signal (Apt^{max}, measured when the

strong background light was added to the measuring light) and the actual photothermal signal (Apt, measured with the modulated exciting light alone), the fraction of absorbed light energy stored in photochemical products (photochemical energy storage) was calculated as $(Apt^{max} - Apt)/Apt^{max}$. Applications of the photoacoustic method to the study of *Synechocystis* PCC6803 and other cyanobacteria can be found in previous publications (e.g. Refs. [26–28]). Chlorophyll fluorescence was measured simultaneously with the photoacoustic signal using a PAM-101 fluorometer (Walz).

2.7. Spectroscopy in vivo

Absorption spectra of cell suspensions were recorded with a Kontron spectrophotometer (Uvikon 942). Chlorophyll fluorescence emission spectra of intact cells, deposited on Millipore nitrocellulose filter (SCWP01300, 8 μm), were measured in liquid nitrogen (77 K) using a Perkin-Elmer LS50B luminescence spectrometer equipped with fiber optics and a home-made low-temperature device. The wavelength of the excitation light was 440 nm.

2.8. Lipid peroxidation measurements

Cellular lipid peroxidation was estimated by determining the amount of malondialdehyde (MDA) adduct with 0.3% of thiobarbituric acid (TBA) according to Vavilin et al. [29]. MDA is a secondary end product of the oxidation of triunsaturated fatty acids that reacts with two molecules of TBA via an acid-catalyzed nucleophilic addition reaction yielding a pinkish-red chromagen with an absorbance maximum at 532 nm [30]. The TBA reactivity was calculated as $(OD_{532}-OD_{600})$ ml^{-1} g^{-1} F.W.

Lipid peroxidation was also monitored by in vivo thermoluminescence measurements, as previously described [31]. *Synechocystis* PCC6803 cells deposited on nitrocellulose filters were slowly heated at a rate of 5 °C min^{-1} from 25 to 160 °C and the thermoluminescence peak at 135 °C was used to detect and quantify membrane lipid hydroperoxides [29,32].

3. Results

3.1. Growth

The quadruple *hli* mutation had little effect on growth of *Synechocystis* PCC6803 in low light (10 μmol photon m^{-2} s^{-1}) (Fig. 1A). In contrast, the WT and the quadruple *hli* mutant behaved differently when cells were grown at a moderately elevated PFD of 100 μmol photon m^{-2} s^{-1}: growth of WT cells was stimulated relative to low-light growth, whereas growth of the quadruple mutant was reduced. This difference was amplified when the PFD was increased further (e.g. to 450 μmol photon m^{-2} s^{-1}), and

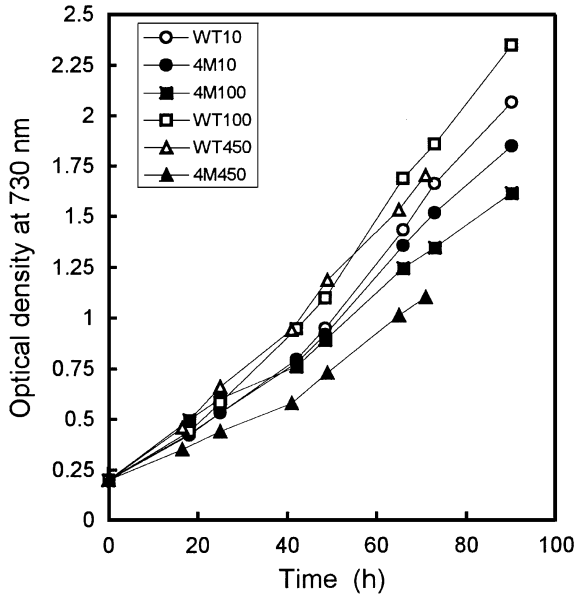

Fig. 1. Growth curves of *Synechocystis* PCC6803 cells. Cell growth was measured at three PFDs: 10, 100 and 450 μmol photon m^{-2} s^{-1}. Wild-type cells = WT and quadruple *hli* mutant cells = 4M. Cell densities were estimated from measurements of the OD of cell suspension at 730 nm.

we determined that, under our experimental conditions, the threshold PFD, above which the quadruple *hli* mutant could not grow at 30 °C, was approximately 500 μmol photon m^{-2} s^{-1}. WT cells were able to grow in this PFD range. These results confirm that the quadruple *hli* mutant exhibits increased photosensitivity relative to WT cells [15].

3.2. Photosynthetic pigments

The quadruple *hli* mutation also had a marked effect on cell pigmentation, particularly at high PFDs (Fig. 2). At 10 μmol photon m^{-2} s^{-1}, mutant cells appeared to be slightly paler than WT cells. However, when the PFD was increased to 200 μmol photon m^{-2} s^{-1}, the quadruple *hli* mutant turned yellow and, at PFDs of 500 μmol photon m^{-2} s^{-1} or above, the cells stopped growing and bleached.

The light-induced changes in cell pigmentation were analyzed by recording the absorption spectra of cell suspensions (not shown). Increasing PFD caused a marked reduction of the chlorophyll and phycobilisome peaks (at 680 and 630 nm, respectively) relative to the absorption in the blue-green region, due in large part to carotenoids. Although this effect was observed for both WT and mutant cells, it was exacerbated in the mutant. Even in low-light-grown cells (10 μmol photon m^{-2} s^{-1}), the amplitude of chlorophyll and phycobilisome peaks was lowered in the mutant relative to the WT.

Photosynthetic pigments were quantified for WT and mutant cells over a broad range of light intensities by HPLC (Fig. 3). Acclimation of WT cells to high PFDs was associated with a marked decrease in the chlorophyll level (Fig. 3A), whereas the total carotenoid level decreased

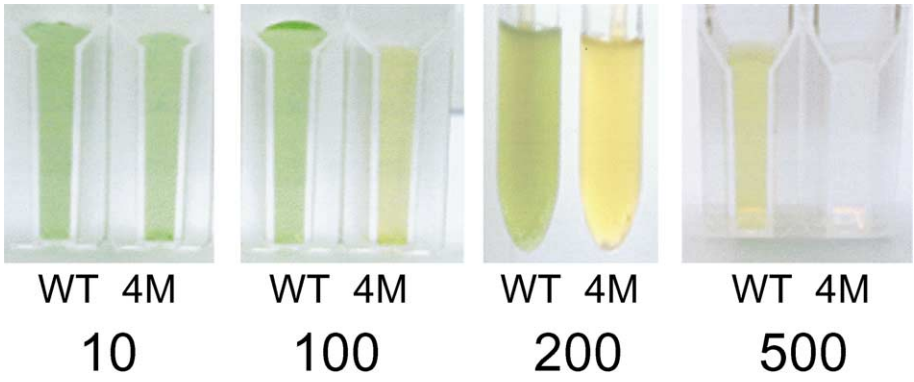

Fig. 2. Effects of light intensity during growth on the pigmentation of *Synechocystis* PCC6803 cells [WT cells and quadruple *hli* mutant cells (4M)] grown at different PFDs: 10, 100, 200 and 500 μmol photon m^{-2} s^{-1}.

slightly (Fig. 3B). However, the relative amounts of the different carotenoids changed, with myxoxanthophyll and, to a lesser extent, zeaxanthin accumulating at the expense of the other carotenoids in high light (Fig. 3C), as observed in other studies (e.g. Ref. [33]). The decrease in chlorophyll with increasing PFD was amplified in the mutant compared to the WT. Even in low light, the mutant exhibited a significant reduction in the amount of chlorophyll per cell relative to WT cells. In contrast to WT cells, the carotenoid level of the mutant initially increased with higher PFD, reaching a maximum level at about 200 μmol photon m^{-2} s^{-1}. The increase in carotenoids at 200 μmol photon m^{-2} s^{-1} was due mainly to a marked accumulation of myxoxanthophyll, which was 2-fold higher in mutant than WT cells. We also observed that the zeaxanthin level in the mutant was higher than that of WT, especially when cells were maintained at PFDs of greater than 200 μmol photon m^{-2} s^{-1}. Thus, the yellow color of the quadruple *hli* mutant grown in high light (Fig. 2) was due to a loss of chlorophyll and phycocyanin along with elevated xanthophyll accumulation. Above 400 μmol photon m^{-2} s^{-1}, there was a general decrease in the pigment content of the mutant that paralleled marked growth inhibition (Fig. 3A and B).

3.3. PSII and PSI levels

The chlorophyll fluorescence emission spectra measured in liquid nitrogen (77 K) in WT and mutant cells deposited on nitrocellulose filters exhibited typical PSII peaks at around 680 and 690 nm and the PSI peak at 720 nm (Fig. 4A and B). Acclimation of WT cells to high PFDs was accompanied by a decrease in PSI relative to the PSII peaks; the F_{720}/F_{690} ratio decreased from approximately 7 in low light to approximately 3 at PFDs >200 μmol photon m^{-2} s^{-1} (Fig. 4C). As chlorophyll *a* is mainly associated with PSI, this spectral change probably reflects the loss of chlorophyll associated with PSI in response to growth in high light. It was previously demonstrated that the PSI/PSII ratio decreases in cyanobacterial cells grown in high light [34], and that this change has photoprotective value since the *pmgA* mutant of *Synechocystis* PCC6803, which cannot modulate its photosystem stoichiometry, was unable to acclimate to long-term exposure to high light [35]. Compared to WT cells, the *hli* quadruple mutant exhibited a noticeably lowered F_{720}/F_{690} ratio, even in low light, in agreement with the pigment data (Fig. 3) in which the mutant strain showed a somewhat reduced chlorophyll level even at relative low light intensities.

A close examination of the 77-K fluorescence spectra measured using the same amounts of WT and mutant cells (Fig. 4A) revealed that the F_{720}/F_{690} decrease in the mutant was mainly due to a decrease in F_{720}, again suggesting lower PSI levels in the mutant. We have determined the abundance of the PSII reaction center protein D1 by Western blotting (Fig. 5). In WT cells, the PSII level was similar in low light (10 μmol photon m^{-2} s^{-1}) and in moderate light (100 μmol m^{-2} s^{-1}). At low light intensity, the D1 abundance in WT and mutant cells was identical while a slight decrease in D1 was noticed at moderately elevated intensity in the mutant relative to WT. Therefore, we conclude that the lowering of the F_{720}/F_{690} ratio in the quadruple *hli* mutant was not a consequence of net accumulation of PSII, but rather reflected a preferential reduction of PSI-associated chlorophylls. This was confirmed by the abundance of the PSI reaction center (PsaA/B) as determined by Western blotting (Fig. 5). In WT cells, PSI decreased with increasing light intensity, as expected. Both in low light and in high light, the quadruple *hli* mutant contained substantially less PSI than the WT.

3.4. Photosynthetic activities

The PSII photochemical efficiency was measured by room-temperature chlorophyll fluorometry in dark-adapted cells (Fig. 6). The $(F_m^{dark} - F_o)/F_m^{dark}$ ratio was significantly higher in the *hli* quadruple mutant (except, however, when cells were grown at PFDs higher than 400 μmol photon m^{-2} s^{-1} where the quantum yield was low in both genotypes). This phenomenon was not due to a differential state transition because the PSII quantum yield of WT cells

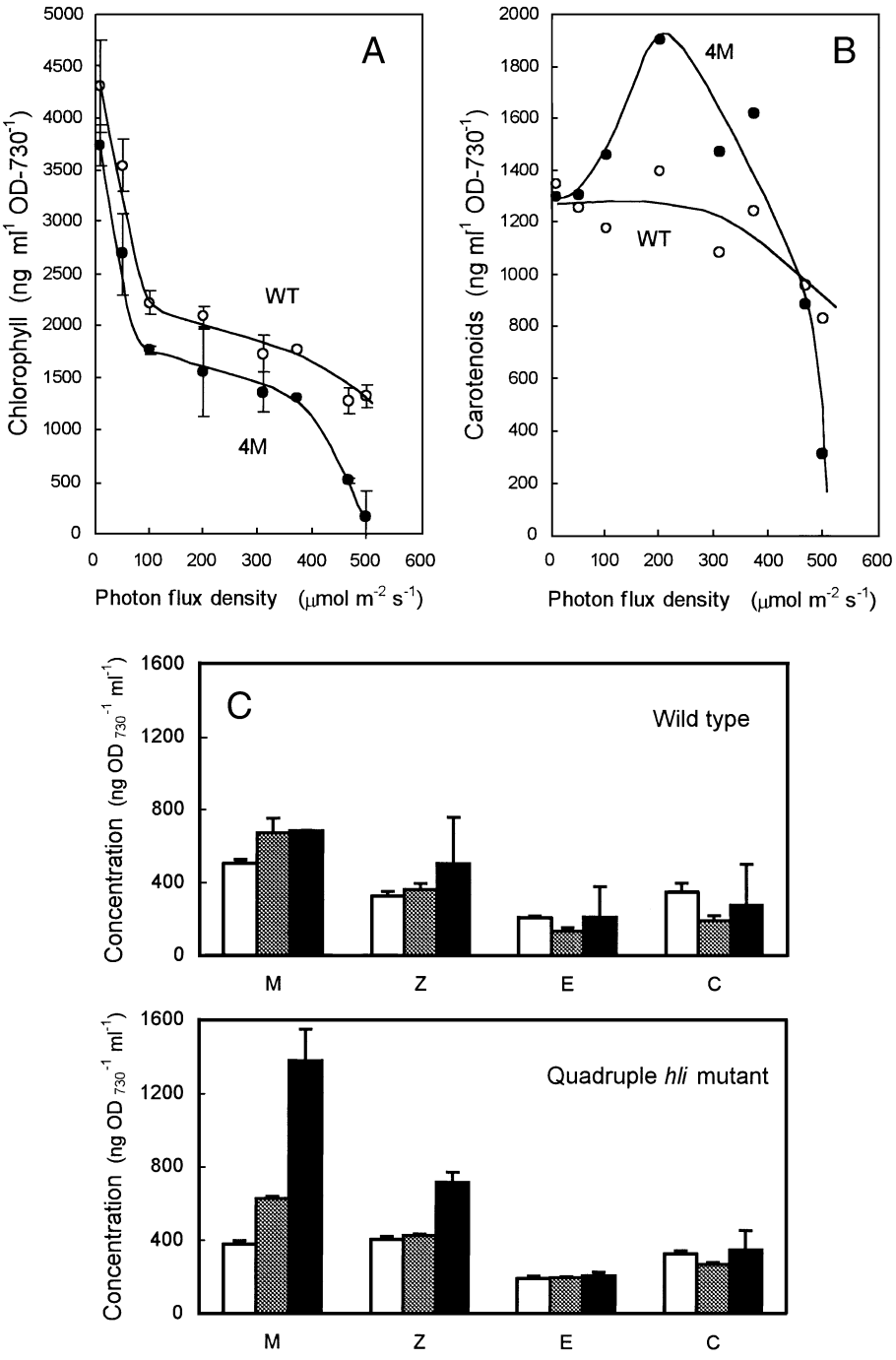

Fig. 3. Effects of light intensity during growth on pigment content and composition of *Synechocystis* PCC6803 cells. Photosynthetic pigments levels ((A) chlorophyll *a* and (B) total carotenoids) were determined in *Synechocystis* PCC6803 cells [WT cells and the quadruple *hli* mutant cells (4M)] grown for 3 days at different PFDs. (C) Carotenoid composition of WT cells and 4M cells grown for 3 days at 10, 100 or 200 μmol photon m^{-2} s^{-1} (white, grey and black rectangles, respectively). Pigment concentrations are expressed per milliliter suspension and per OD_{730}. OD_{730} can be easily converted into cell numbers using the conversion factors given in Section 2.1. M = myxoxanthophyll; Z = zeaxanthin; E = echinenone; C = β-carotene.

remained low compared to the mutant when cells were driven to state 1 in the presence of DCMU (Table 1). Also, lowering of the F_{720}/F_{690} ratio measured in the quadruple mutant in liquid nitrogen was not cancelled when the cells were in state 1. The PSII quantum yield was also measured in the light as $(F'_m - F_s)/F'_m$ (Fig. 7). Compared to WT cells, mutant cells exhibited an increased quantum yield over the entire range of PFDs examined, and this difference was particularly marked in high-light-grown cells. The room-temperature chlorophyll fluorescence measurements suggest that the PSII photochemical efficiency and linear electron transport were stimulated in the mutant relative to

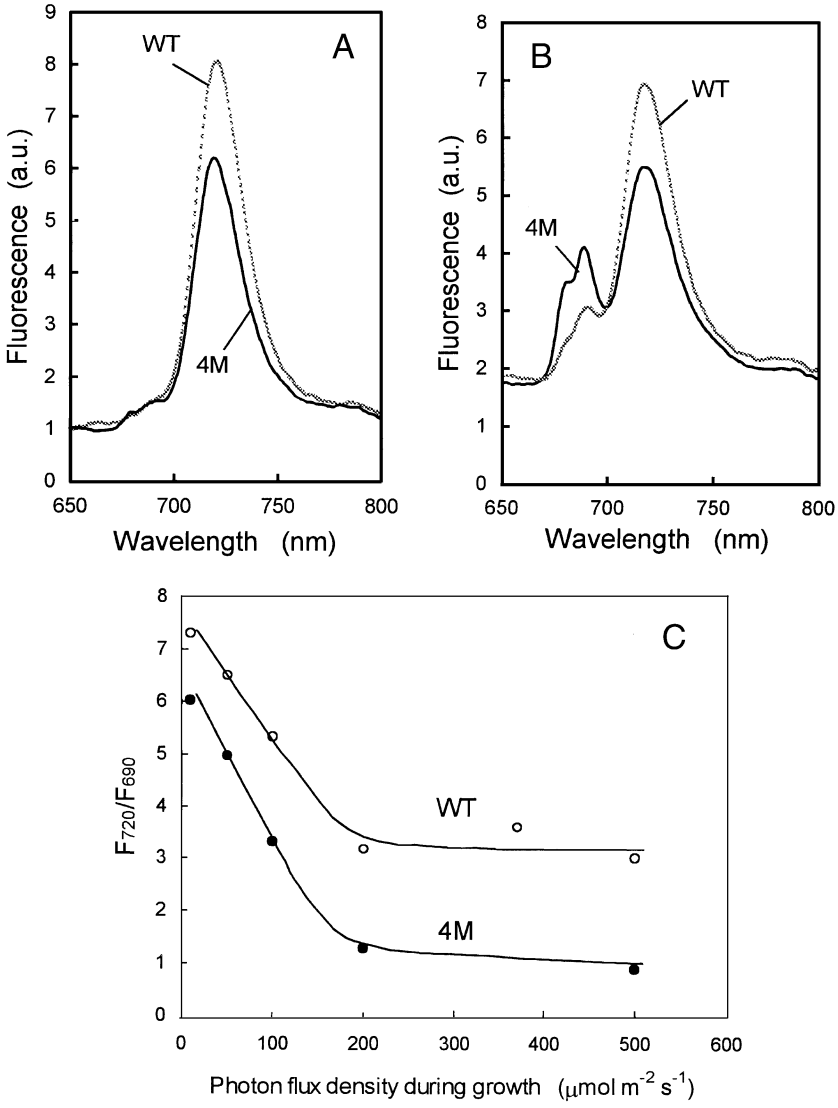

Fig. 4. Effects of the quadruple *hli* mutation on PSI and PSII levels. (A) Seventy-seven kelvin fluorescence emission spectra of WT and 4M cells grown at 100 μmol photon m^{-2} s^{-1}. The same amount of cells, as measured by the OD_{730}, was deposited on a filter. The spectra were not normalized. Wavelength of excitation light: 440 nm. (B) Seventy-seven kelvin fluorescence emission spectra of WT and 4M cells grown at 210 μmol photon m^{-2} s^{-1}. Excitation light: 440 nm. (C) Effects of light intensity during growth on the ratio of 77 K chlorophyll fluorescence peaks at 720 nm (F_{720}) to 690 nm (F_{690}) in *Synechocystis* PCC6803 cells. WT = wild-type cells and 4M = quadruple *hli* mutant cells.

WT. However, because of the change in PSI/PSII stoichiometry (Figs. 4 and 5), the contribution of PSI to the F_o fluorescence level is probably lower in the quadruple *hli* mutant, and this can cause an apparent increase in the F_v/F_m ratio which is not directly related to a change in PSII photochemical efficiency.

Because of the limitation of the fluorometric estimation of photosynthetic electron transport efficiency in the case where the F_o level contains variable PSI contribution, photosynthetic O_2 evolution was measured with a Clark-type O_2 electrode at various PFDs (Fig. 8). The results confirm the fluorescence measurements: photosynthetic electron transport activity was higher in the mutant. This was particularly striking at high, saturating light intensities and was enhanced in high-light-grown cells. In limiting light conditions, O_2 evolution was also enhanced in the mutant relative to WT cells, but the difference was less marked. A lower rate of linear electron transport with more PSI in WT cells (vs. mutant cells) could suggest that PSI is partially involved in cyclic electron transport.

In vivo chlorophyll fluorescence measurements were also performed in the presence of PSII electron acceptors (Table 2). The use of these acceptors allows the efficiency of PSII-mediated electron transport to be measured independently of PSI; addition of an excess PSII electron acceptor will cancel the stoichiometric imbalance between PSII and PSI which is more pronounced in WT than in the mutant (Fig. 4). As shown in Table 2, the increased electron transport efficiency

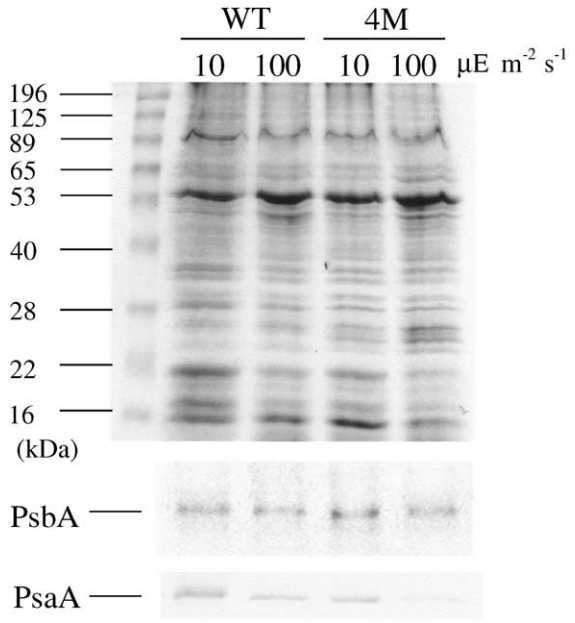

Fig. 5. Western blot of D1 (PsbA) and PsaA/B in WT and 4M cells grown at 10 or 100 μmol photon m^{-2} s^{-1}. Fifteen micrograms per lane of proteins was loaded on the gels. The Coomassie blue-stained gel shown on the top confirms equal loading.

in mutant cells relative to WT cells was not cancelled by DCBQ. Because DCBQ is also a fluorescence quencher which could interfere with the estimation of electron transfer from chlorophyll fluorescence signals [36], we also used

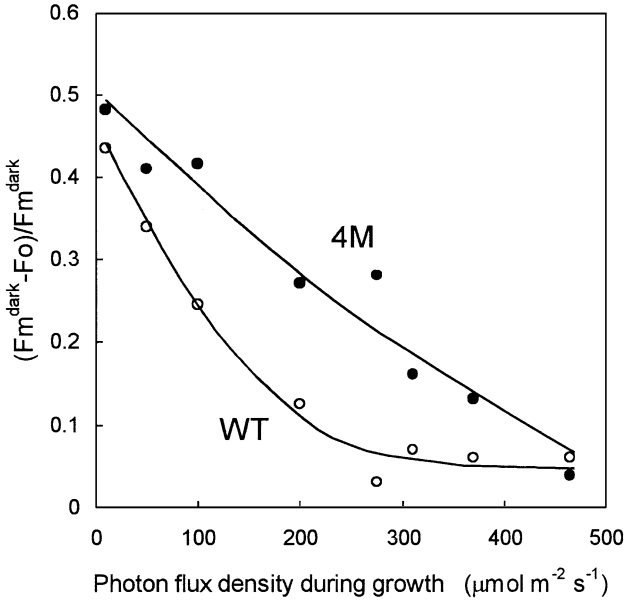

Fig. 6. Effects of light intensity during growth of the PSII quantum efficiency in *Synechocystis* PCC6803 cells. The PSII quantum yield was measured as $(F_m^{dark} - F_o)/F_m^{dark}$ after dark adaptation of the cell cultures. WT = open circles and quadruple *hli* mutant (4M) = closed symbols. Cells were grown for 3 days at the different PFDs.

Table 1
Quantum yield of PSII photochemistry and F_{720}/F_{690} 77 K chlorophyll fluorescence ratio in *Synechocystis* PCC6803 cells adapted to the dark or driven to state 1

Strains and growth conditions	In the dark		In state 1	
	$(F_m^{dark} - F_o)/F_m^{dark}$	F_{720}/F_{690}	$(F_m - F_o)/F_m$	F_{720}/F_{690}
10 μmol photon m^{-2} s^{-1}				
WT	0.488 ± 0.009	n.d.	0.507 ± 0.003	n.d.
4M	0.518 ± 0.024	n.d.	0.574 ± 0.006	n.d.
100 μmol photon m^{-2} s^{-1}				
WT	0.192 ± 0.025	1.34 ± 0.01	0.395 ± 0.017	1.03 ± 0.04
4M	0.360 ± 0.015	1.12 ± 0.03	0.526 ± 0.007	0.65 ± 0.01

WT cells and quadruple *hli* mutant cells (4M) grown in low light (10 μmol photon m^{-2} s^{-1}) or in high light (100 μmol photon m^{-2} s^{-1}) were dark adapted for a minimum of 30 min or were driven to state 1 by illumination with low-intensity white light in the presence of DCMU (40 μM), as described by Campbell et al. [22]. The PSII quantum yield was measured by chlorophyll fluorometry as $(F_m - F_o)/F_m$. N.d. = not determined. Data are mean values of four experiments ± S.D.

DAD (Table 2) and DBMIB at relatively high concentrations (150–200 μM), which is both a PSII electron acceptor and an inhibitor of cyt b_6/f (data not shown). In both cases, the increase in electron transport activity, as measured by chlorophyll fluorometry, in the mutant relative to WT, was preserved, and this cannot be attributed to a change in the concentration of PSII because the fluorescence parameter $(F'_m - F_s)/F'_m$ is indicative of the *efficiency* of PSII-mediated electron transfer.

The preservation of high photosynthetic activities in the mutant indicates that the pigment losses were not associated with appreciable perturbations of reaction center and photosynthetic electron transfer function. Nevertheless, growth of the quadruple mutant in high light was reduced compared to that of the WT (Fig. 1). Clearly, growth inhibition and pigment change must be ascribed to factors other than the direct inhibition of photosynthetic electron transport. It is possible that the effect on growth is a consequence of chronic oxidative stress in the mutant (however, see below) or a perturbation in gene expression/protein synthesis.

The photoacoustic technique measures the conversion of light energy to heat in an absorbing sample and hence the storage of light energy in photochemical products [25]. Photoacoustic measurements of photochemical energy storage in weak red light exciting both photosystems (Fig. 9) confirmed that photochemistry associated with both PSI and PSII was more efficient in the mutant strain. Energy storage was higher in the mutant over the whole range of PFDs used during growth (except at PFDs close to 500 μmol photon m^{-2} s^{-1}), that is, less energy was dissipated as heat in the mutant. This phenotype of the mutant was even more clearly observed when thermal deactivation of excited pigments was measured in layers of WT and mutant cells containing identical amounts of chlorophyll (Fig. 10). Heat emission in WT cells was enhanced by approximately 7.5% compared to

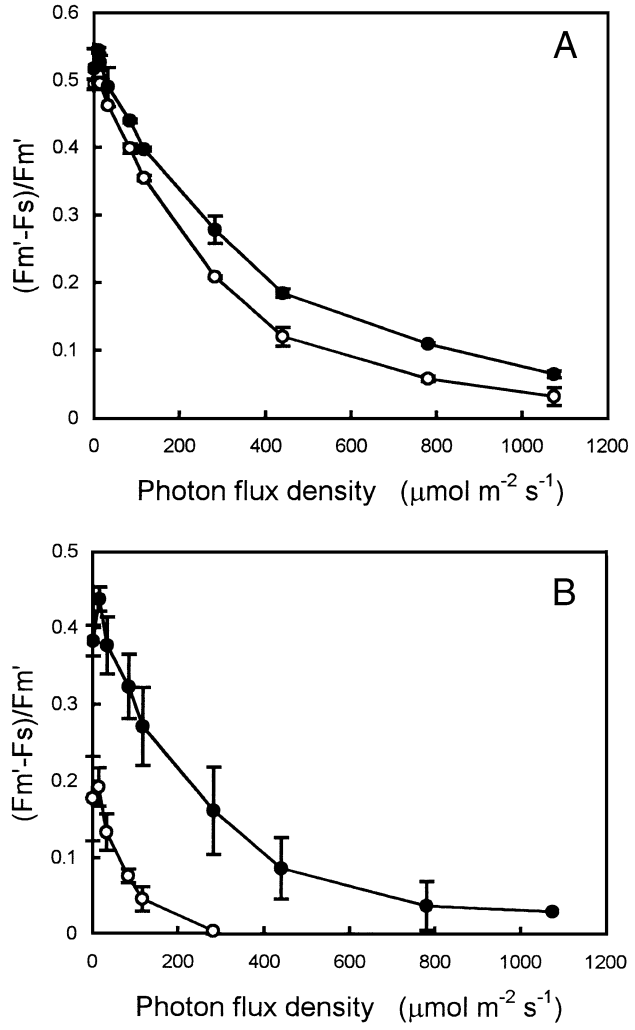

Fig. 7. Light dependence of the quantum yield of PSII photochemistry in *Synechocystis* PCC6803 cells. The actual quantum yield was measured as $(F'_m - F_s)/F'_m$ in illuminated cells. WT cells (open circles) and quadruple *hli* mutant cells (4M, closed symbols) were grown for 3 days at a PFD of (A) 10 μmol photon m^{-2} s^{-1} or (B) 100 μmol photon m^{-2} s^{-1}.

the heat emission signal of the mutant. This result suggests that there is an altered efficiency of excitation energy transfer from the chlorophyll antennae of PSII to the reaction center; excitation transfer is more efficient in mutant than WT cells. Chlorophyll fluorescence emission was also decreased in the quadruple *hli* mutant but the interpretation of this change in terms of nonphotochemical energy dissipation in PSII is difficult because of the lower contribution of PSI to the F_o fluorescence.

3.5. Photoinhibition and photooxidation during high-light stress in the short term

Cells grown for 4 days at a PFD of 100 μmol photon m^{-2} s^{-1} were suddenly exposed to a very high PFD of 900 μmol photon m^{-2} s^{-1}. Fig. 11 shows the photosynthetic activity of the cells, as measured by photo-induced O_2 production (on a cell unit basis), after different times of exposure to the high-light stress conditions. As shown in Fig. 8, photosynthetic O_2 evolution by mutant cells was higher than that measured in WT cells. During the first 5 h of illumination, O_2 evolution decreased rapidly in the mutant so that the O_2 evolution activity of both strains became roughly identical. Subsequently, the rate of high-light-induced decrease in O_2 evolution appeared to be similar in both strains. Consequently, after 1 day exposure to high light, photosynthesis was noticeably photoinhibited but no difference was found between the WT and the quadruple *hli* mutant. This treatment was also associated with a dramatic modification of the coloration and pigment content of mutant cell suspensions, which turned yellow (not shown). The MDA level was not increased by the high-light stress: The TBA reactivity [expressed in $(OD_{532} - OD_{600})$ ml^{-1} g^{-1}] was 0.22 ± 0.03 and 0.20 ± 0.02 in the WT before and after high-light stress and 0.28 ± 0.04 and 0.24 ± 0.04 in the

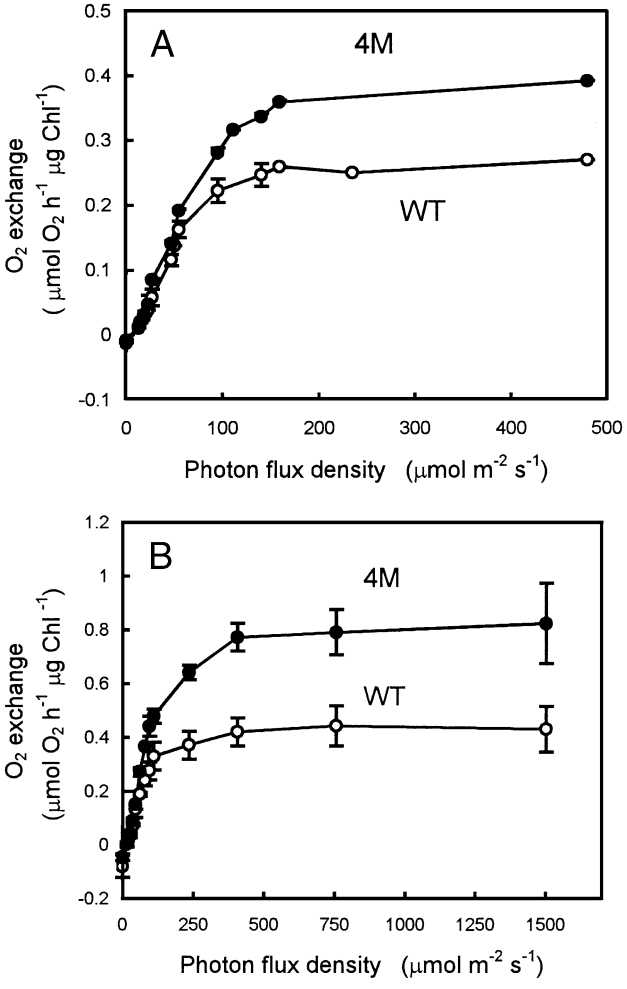

Fig. 8. Light dependence of net O_2 exchange by *Synechocystis* PCC6803 cells. WT cells (open circles) and quadruple *hli* mutant cells (4M, closed circles) were grown for 3 days at a PFD of (A) 10 μmol photon m^{-2} s^{-1} or (B) 100 μmol photon m^{-2} s^{-1}.

Table 2
Quantum yield of PSII-mediated electron transport in *Synechocystis* PCC6803 cells incubated with PSII electron acceptors

Strains and growth conditions	PFD (μmol photon m^{-2} s^{-1})		
	0	70	140
10 μmol photon m^{-2} s^{-1}			
WT + DCBQ	0.401 ± 0.049	0.252 ± 0.029	0.160 ± 0.033
Mutant + DCBQ	0.504 ± 0.028	0.339 ± 0.035	0.208 ± 0.026
WT + DAD	0.454 ± 0.004	0.370 ± 0.003	0.313 ± 0.011
Mutant + DAD	0.519 ± 0.019	0.415 ± 0.009	0.331 ± 0.042
100 μmol photon m^{-2} s^{-1}			
WT + DCBQ	0.208 ± 0.052	0.147 ± 0.015	n.d.
Mutant + DCBQ	0.286 ± 0.048	0.183 ± 0.011	n.d.
WT + DAD	0.270	0.158	0.134
Mutant + DAD	0.335	0.233	0.206

The $(F'_m - F_s)/F'_m$ ratio was measured in WT and mutant *Synechocystis* cells illuminated in the presence of 3.75 mM $K_3Fe(CN)_6$ and 300 μM DCBQ or 600 μM DAD. Cells were grown in low light (10 μmol photon m^{-2} s^{-1}) or in high light (100 μmol photon m^{-2} s^{-1}). N.d. = not determined. Data are mean values of three experiments ± S.D. except the DAD experiments on high-light-grown cells which were repeated two times.

mutant before and after stress, respectively. Thus, the pigment change observed in the mutant was not associated with extensive lipid peroxidation. This observation was confirmed by estimating photooxidative damage using thermoluminescence measurements at high temperature, as described previously [31]. Accumulation of lipid hydroperoxides in photosynthetic membranes is known to be accompanied by the appearance of a thermoluminescence band at around 135 °C, which can be used as an index of lipid peroxidation [29,31,32]. The results confirmed the MDA data: no significant increase in lipid peroxidation-related thermoluminscence signal occurred in

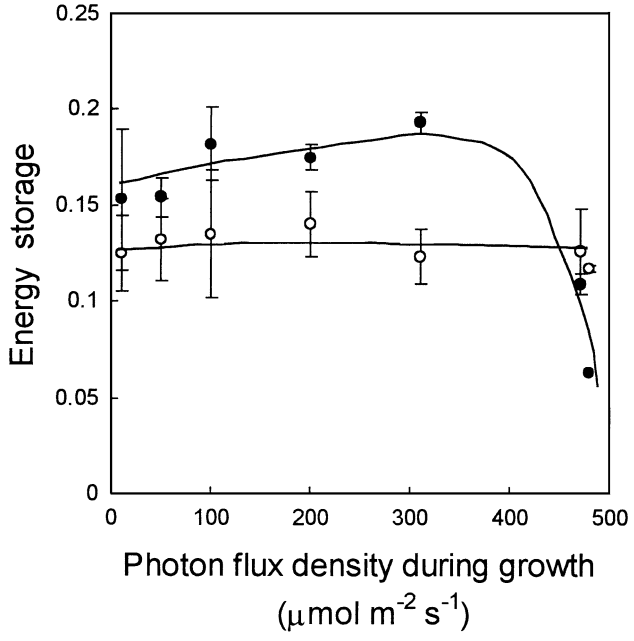

Fig. 9. Photoacoustic measurements of photochemical energy storage in *Synechocystis* PCC6803 cells. WT = open symbols and quadruple *hli* mutant = closed symbols. Photoacoustic measurements were performed in red light of PFD 20 μmol photon m^{-2} s^{-1}. Cells were grown for 3 days at different PFDs ranging from 10 to 500 μmol photon m^{-2} s^{-1}.

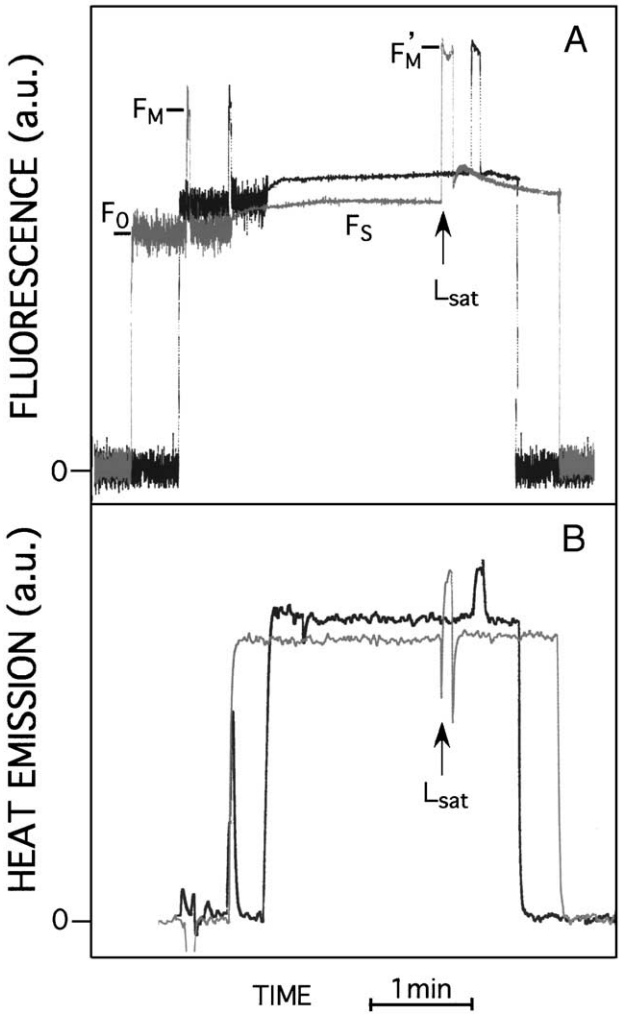

Fig. 10. Nonphotochemical energy dissipation pathways in *Synechocystis* PCC6803 cells. (A) Chlorophyll fluorescence and (B) heat emission were measured simultaneously in *Synechocystis* PCC6803 cells [WT cells in black and quadruple *hli* mutant cells (4M) in grey] grown at 100 μmol photon m^{-2} s^{-1} for 4 days. The initial level of chlorophyll fluorescence (F_o) was induced by a weak red light and the maximal level (F_m) was obtained with an 800-ms pulse of intense white light. Cells were then illuminated with an actinic red light of PFD 18 μmol m^{-2} s^{-1} modulated at 10 Hz, leading to the steady-state fluorescence level (F_s) and the steady-state emission of heat. Application of an intense white light (Lsat) yielded the maximal levels of fluorescence (F'_m) and heat emission. Measurements were performed on samples with identical amounts of chlorophyll so that the amount of light energy absorbed by the samples was comparable. This is confirmed by the amplitude of the maximal (light-saturated) heat emission (proportional to the light absorption) which was similar in WT and 4M samples. This normalization to the chlorophyll content was necessary to compare absolute levels of fluorescence and heat emission.

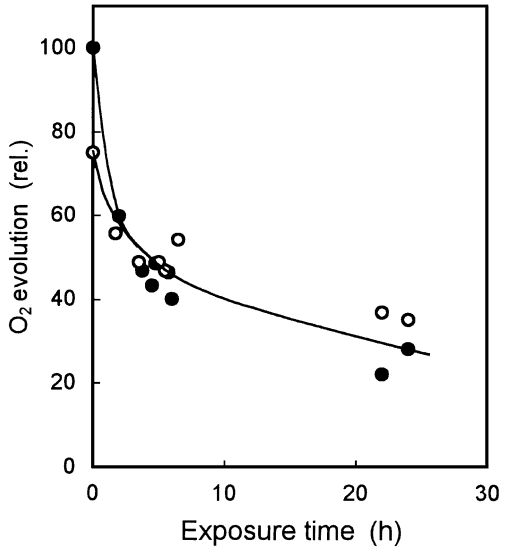

Fig. 11. Decrease in photosynthetic O_2 evolution in *Synechocystis* PCC6803 cells suddenly exposed to a strong light stress. *Synechocystis* PCC6803 cells [WT cells (open symbols) and quadruple *hli* mutant cells (closed symbols)] were transferred from 100 to 900 μmol photon m^{-2} s^{-1}. Net O_2 evolution was measured on a cell unit basis with white light of PFD 150 μmol photon m^{-2} s^{-1}. One hundred percent = 0.016 μmol O_2 min^{-1} ml^{-1} OD_{730}^{-1}. This value corresponds to 0.54 μmol O_2 h^{-1} μg $chlorophyll^{-1}$.

Synechocystis cells at 900 μmol photon m^{-2} s^{-1} (not shown). Thus, one can conclude that, at least in short-term experiments, the quadruple mutant cells do not seem to be much more sensitive to photooxidation or photoinhibition than the WT.

4. Discussion

This work has shown that HLIPs have an important physiological function related to light acclimation. Indeed, the responses of *Synechocystis* PCC6803 to the light environment were dramatically modified in the strain inactivated for four of the five *hli* genes. In particular, the quadruple mutant was much more affected by high light intensities than the WT: growth of HLIP-deficient mutant cells was noticeably inhibited at PFDs higher than about 400 μmol photon m^{-2} s^{-1} and cell growth stopped at about 500 μmol photon m^{-2} s^{-1}. At lower PFDs, the mutant tended to 'overreact' to a rise in light intensity. Photoacclimation of WT *Synechocystis* cells involved primarily a decrease in their light-harvesting capacities, as reflected by a decrease in the level of chlorophyll and phycobilisomes. These light-induced changes appeared dramatically amplified in quadruple *hli* mutant. Compared to WT cells, the mutant contained noticeably less chlorophyll, whereas the carotenoid concentration was markedly increased. This suggests that the HLIP-deficient strain sensed greater excitation pressure than WT at any PFD, which may have triggered amplification of protective responses including modification of cellular pigment content and alterations in photosystem stoichiometry. However, some of the pigment changes were not congruent with changes observed in high-light-grown WT cells: myxoxanthophyll increased to higher levels than ever observed in WT cells. The mutant also appeared to be capable of considerable photoprotection because their photosynthetic capacity was not strongly inhibited over a rather large range of PFDs (up to 400 μmol photon m^{-2} s^{-1}), although the growth rate did decline significantly.

Although HLIPs are generally considered to be stress proteins that accumulate under conditions of excess light energy [8,11,15], low amounts of all four Hli polypeptides were detected in *Synechocystis* cells maintained in low light and during optimal growth conditions [15]. Similarly, Funk and Vermaas [14] found high levels of HLIP transcripts in a PSI-less *Synechocystis* mutant in low light, when the cells were not experiencing light stress (although the PQ pool could still be in a highly reduced state), and Jansson et al. [8] detected HLIP transcripts in low-light-grown *Arabidopsis* plants. The presence of HLIPs in low light is consistent with the results that, even at low PFD (10 μmol photon m^{-2} s^{-1}), the quadruple *hli* mutant and WT differ to some extent with respect to pigment content/composition and photochemical activity.

The phenotype of the quadruple *hli* mutant suggests, in general, that it is in a state in which a given amount of absorbed light energy systematically represents greater excess absorption than in WT cells. Therefore, the presence of the HLIPs would help cells, either through a direct or indirect means, to eliminate excitation energy. This hypothesis is consistent with much of the functional data presented in this paper. Strikingly, the quadruple *hli* mutant exhibited enhanced photochemical activities, as probed by fluorescence, photoacoustic and O_2 evolution measurements (Figs. 6–9), relative to WT cells over a range of light conditions. Light that is absorbed by the photosystems and not used for photochemical work is dissipated either as emitted light (fluorescence) or heat. The presence of HLIPs in WT cells stimulated thermal deactivation of excited pigments (Fig. 10). Thus, this comparative analysis of WT and *hli* mutant cells suggests that HLIP synthesis is associated with increased dissipation of absorbed excitation energy by non-photochemical pathways over a range of light conditions; this dissipation of excitation energy is absent in the quadruple *hli* mutant.

The increase in photon energy dissipation in the WT compared to the quadruple *hli* mutant can be a direct effect of the interaction of HLIPs with the photosystems or it can result indirectly from the modification of the composition and/or organisation of the photosynthetic complexes in mutant cells. However, there are no obvious differences in photosynthetic complexes that readily explain the functional differences observed. Differences in photochemical activity between WT cells and the quadruple mutant cannot be

attributed to differences in the light-harvesting antenna size. Indeed, a decrease in antenna pigments would have resulted in a lower, not higher O_2 production upon exposure of the cells to limiting light conditions. Conversely, an increase in pigment antenna size would have led to a more rapid saturation of photosynthesis with light intensity, which was not observed. Moreover, the increased O_2 evolution in the mutant cannot be attributed to a net increase in the PSII level (Fig. 5). There was also the possibility that photosynthesis was enhanced in the mutant as a result of an increased PSII/PSI ratio. In WT cells, the PSII/PSI ratio was very low. A more balanced ratio of PSI and PSII in the mutant could result in increased linear electron flow and less energy dissipation as heat. However, an increase in PSII-mediated electron transport efficiency in the quadruple *hli* mutant relative to WT cells was still observed when PSI was bypassed by using PSII electron acceptors (Table 2). Nevertheless, it is difficult to determine whether the HLIPs have a direct or indirect affect on the utilization of absorbed light energy. Indirect effects may occur if the absence of the HLIPs alters excitation transfer between the chlorophyll *a* antennae and PSII reaction centers. Organisation changes within the photosynthetic pigment beds may occur if the HLIPs, or some of them, function in the control of chlorophyll biosynthesis and the binding of intermediates in this pathway, as suggested by Xu et al. [16].

The absence of HLIP in *Synechocystis* resulted neither in extensive photooxidative damage nor in a stimulated photoinhibition when cells were suddenly exposed to a relatively high light intensity (Fig. 11). Thus, HLIPs do not seem to have a direct protective effect against cell photodestruction. It is conceivable that one of the primary effects of HLIP-dependent energy dissipation is to regulate the reduction state of the PQ pool and to decrease electron pressure on the photosynthetic electron transport chain, which in turn would influence the responses of the cell to environmental light conditions. Indeed, the redox state of the plastoquinone pool is believed to play a key role in the regulation of gene expression [37,38]. Similarly, long-term exposure of NPQ-deficient *Arabidopsis* mutants to (constant) high light did not result in enhanced photoinhibition of PSII, but altered regulatory responses that influence the Lhc levels or the accumulation of antioxidants [31].

The xanthophyll cycle-based energy dissipation that dominates in vascular plants [39–41] has features that clearly distinguish it from the HLIP-dependent processes reported here. Photosystems of HLIP-containing cells seem to be maintained in an energy-dissipative state that occurs both in low and high light. In vascular plants, the majority of light harvesting and energy dissipation occurs in the same antenna polypeptides, and, because of the dynamic response of xanthophyll cycle-based energy dissipation to the transthylakoid pH gradient, significant thermal dissipation only occurs when plants experience excess excitation. However, under some stress conditions, vascular plants can exhibit persistent thermal dissipation of excess absorbed photons [42–46]. Öquist et al. [47] ascribed the persistent lowering of PSII photochemical efficiency in high-light-treated leaves to a stable down-regulation of PSII reaction centers, which can replace part of the regulation exerted by ΔpH-dependent NPQ. Long-term exposure of green algae to excess light energy induced a form of NPQ which is less dependent on ΔpH and which could require an ELIP-type polypeptide [48]. From our results and from the characteristics of sustained NPQ, we raise the possibility that some aspects of this quenching in stressed vascular plants may be related to HLIP function. Concomitant analysis of ΔpH-independent energy dissipation and HLIP levels in plants exposed to various environmental stresses should help clarify this point.

If the HLIPs do play a direct role in quenching, they may exert their affect on the PSII core reaction center. Reaction center quenching is expected to lead to a reduction of both limiting and saturating levels of O_2 evolution, as observed for HLIP-dependent energy dissipation (Fig. 9). In contrast, elimination of excitation energy within the pigment bed would be expected to result in lower efficiency of O_2 evolution in low light with a similar maximal activity.

A role as chlorophyll buffer polypeptides was also suggested for the HLIPs. Such buffer polypeptides would minimize photodestructions by free chlorophylls during turnover of photosynthetic complexes in high light [14]. Although we cannot exclude this possibility, it is not supported by the finding that the quadruple *hli* mutant is not more sensitive to photooxidative damage of PSII and does not exhibit increased lipid peroxidation relative to WT cells. However, if the HLIPs are involved in chlorophyll biosynthesis (as recently suggested by Xu et al. [16]) and the generation of pigment protein complexes, the phenotype of the quadruple *hli* mutant may reflect a role for these polypeptides in the establishment of functionally normal pigment protein complexes that properly adjust as environmental conditions change.

5. Conclusions

Because HLIPs and PSII-S are thought to have evolved earlier than the Lhc polypeptides [8,9,17,49], light harvesting, as performed by Lhc polypeptides, may represent a relatively recent addition to the functions encoded by this extended gene family. Presumably, progressive colonization of terrestrial environments by plants was associated with the need to acclimate to high light, shaded habitats and fluctuating light conditions. The dual function of energy quenching and energy transfer occurring in a single protein complex might thus represent an important evolutionary step that has made the Lhc polypeptides superior to phycobilisomes/HLIPs for photosynthesis in a terrestrial environment. Possibly, HLIPs are primitive Lhc-type polypeptides that function in the establishment of core reaction center complexes competent for energy dissipation (by either direct or indirect mechanisms). However, the preservation of

HLIPs in vascular plants [8] suggests that their function may still be important for light stress resistance and that they may serve a role that is complementary to that fulfilled by the xanthophyll cycle. Possibly, this role is related to persistent lowering of the photochemical efficiency associated with long-term acclimation of plants to excess light energy, while xanthophyll cycle-based energy dissipation represents a rapid mechanism for short-term adjustment of light harvesting to light intensity fluctuations.

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