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A Cyanobacterial Gene Family Coding for Single-Helix Proteins Resembling Part of the Light-Harvesting Proteins from Higher Plants[†]

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ABSTRACT: In the cyanobacterium *Synechocystis* sp. PCC 6803 five genes were identified with significant sequence similarity to regions of members of the eukaryotic chlorophyll *a*/*b* binding gene family (Cab family) and to *hli*A, a gene coding for a small high-light-induced protein in *Synechococcus* sp. PCC 7942. Four of these five genes are 174−213 bp in length and code for small proteins predicted to have a single transmembrane helix. The fifth Cab-like gene in *Synechocystis* sp. PCC 6803 is much longer and codes for a protein of which the N-terminal 80% resemble ferrochelatase but the C-terminal domain has similarity to Cab regions. The small genes were expressed preferentially in the absence of photosystem I, but gene expression was not significantly enhanced at moderately high light intensity. Therefore they were not designated as *hli* (high-light-induced) as was done for the *Synechococcus* sp. PCC 7942 homolog. Instead, the genes have been named *scp*, as the corresponding polypeptides of *Synechocystis* sp. PCC 6803 are small Cab-like proteins (SCP). The *scp*A gene, which codes for ferrochelatase with a C-terminal Cab-like extension, was interrupted by the insertion of a kanamycin-resistance cassette between the ferrochelatase and Cab-like gene domains. In the PS I-less background, interruption of *scp*A was found to lead to increased tolerance to high light intensity and to the requirement of a slightly higher light intensity to drive photosystem II electron transfer, suggestive of decreased light-harvesting efficiency in the absence of the C-terminal extension of ScpA. Immunodetection of ScpC and ScpD indicated that either or both accumulated in PS I-less strains. These proteins were also detected in bands of more than 45 kDa on denaturing gels, raising the possibility that they may occur as stable oligomers. The SCPs represent a new group of cyanobacterial proteins that, in view of their primary structure and response to deletion of photosystem I, are likely to be involved in transient pigment binding.

The two photosystems, photosystem II (PS II)[1] and photosystem I (PS I), that are found in plants and cyanobacteria together catalyze oxygenic photosynthesis. Each of the photosystems consists of a reaction center, where light-induced charge separation takes place, and of an antenna system that feeds excitations into the reaction center complex. Even though the reaction centers are remarkably conserved throughout evolution, different types of organisms have adopted and retained different antenna pigment−protein complexes. In higher plants, the most abundant peripheral antenna is the chlorophyll *a*/*b*-binding light-harvesting complex (referred to as LHC) (*1*, *2*), which is encoded by a multi-gene family of at least 10 different *cab* genes.

All chlorophyll *a*/*b*-binding proteins (Cab proteins) in eukaryotes are related to each other according to their protein sequence and are assumed to share a common evolutionary origin. The *cab* gene family is nuclear-encoded, and most members code for light-harvesting chlorophyll proteins (LHC) that have three membrane-spanning regions per polypeptide. However, the family also includes the gene for the PS II−S protein, which is predicted to have four membrane-spanning regions (*3*, *4*), as well as for early light-induced proteins (ELIPs), which bind chlorophyll *a* and lutein (*5*). More distant relatives are the fucoxanthin−chlorophyll *a*/*c*-binding proteins of the chromophytic algae (*6*). For all members of the Cab family the first helix is similar to the third one, and the second helix similar to the fourth (if present). The structural determination of LHCII (*1*) has shown that the first and third membrane-spanning helices are held together by Glu−Arg ion pairs and that two luteins form an internal cross-brace in the center of the complex, providing direct, strong links between the helices.

In contrast to higher plants, cyanobacteria lack chlorophyll *b* as well as multi-helix Cab proteins. The major peripheral light-harvesting complex is the phycobilisome (*7*), which is associated with the thylakoid membrane and contributes to the deep blue−green color of cyanobacteria. However, prochlorophytes, which contain chlorophyll *b* and are closely related evolutionarily to cyanobacteria (*8*), have chlorophyll *a* and chlorophyll *b* as their peripheral antenna and lack

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[1] Abbreviations: PS I, photosystem I; PS II, photosystem II; LAHG, light-activated heterotrophic growth.

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phycobilisomes. The peripheral antenna proteins in prochlorophytes have no apparent evolutionary relationship to Cab proteins but are similar to PS II core antenna proteins (*9*).

A small protein of 8 kDa has been identified in the cyanobacterium *Synechococcus* sp. PCC 7942 (to be referred to as *Synechococcus* 7942 here). This protein is predicted to have a single membrane-spanning helix and shows significant sequence similarity to the first and third membrane-spanning region of proteins belonging to the Cab family (*10*). This small cyanobacterial protein was designated a high light-inducible protein (HLIP) since its RNA level was found to increase after transfer of cells to high light. Deletion of the corresponding gene (*hli*A) had no effect on the growth or phenotype of the bacterium. The similarity of this protein to the members of the Cab family suggests that the former is a single-helix relative of the latter.

Here we report the existence of five genes and the presence of corresponding transcripts that are predicted to code for small Cab-like proteins (SCPs) in the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis* 6803). These SCPs are predicted to have a single membrane span with sequence similarity to the first and third transmembrane regions in Cab-like proteins. Whereas four of these proteins have a size comparable to HliA, the gene for the fifth SCP codes for ferrochelatase and carries a sequence coding for a short Cab-like polypeptide at its 3′ end.

MATERIALS AND METHODS

Growth Conditions. Synechocystis 6803 strains of wild type and the PS I-less (*psa*AB$^-$; ref *11*), PS II-less (*psb*DIC$^-$/*psb*DII$^-$; ref *12*), PS I-less and PS II-less (PS I-less/*psb*DIC$^-$/*psb*DII$^-$; ref *13*), and PS I-less/*chl*L$^-$ (*14*) mutants were cultivated at 30 °C in BG-11 medium (*15*). PS I-less and PS II-less mutants were provided with 15 mM glucose. Growth curves of wild type and the *scp*A interruption mutant were performed in BG-11 medium under photoautotrophic conditions (in the absence of glucose) at 50 or 30 μE m^{-2} s^{-1}. The liquid cultures were started at an OD$_{730}$ of 0.05, and samples were taken every 12 h.

RNA Preparation and Northern Hybridization. Expression of the *scp* genes at the RNA level was tested by Northern blotting. Primers used for PCR amplification were the following: *scp*A, CGA CGC CTT GGC CCA GAT GG and GCA AGC CGA CAA AAT GCA ACG; *scp*B, GTT GCC TTT TTC ACC ACA GA and CAG AAT GCC GAA GAA GTG AAG; *scp*C, CAT AGA CTT TTA CTA GGA GTA ATC and CAG GCT ATT TAA CCA ACC AAT GA; *scp*D, CTC AAA TAC CCA ATC AAG GAG and GAG CAA CCA ACC CAC AAT GCC; and *scp*E, CTA AGT TAA TTA TCC CAG GAA ATC C and GCG GAC TCC CAA CCA GGC C. SCP genes (*scp*) correspond to the following nucleotide numbers of the *Synechocystis* 6803 genomic sequence as they are accessible through CyanoBase: *scp*A, 2849096−2850259; *scp*B, 1142015−1141803; *scp*C, 701350−701138; *scp*D, 982968−983180; and *scp*E, 398188−398361. Each of the primers recognized unique sequences within the coding region of the corresponding gene. For total RNA isolation, 50−100 mL of liquid culture in the exponential phase was harvested and the preparation was done according to (*16*). RNA was separated according to ref *17*.

Functional Deletion of the SCP Part in the Ferrochelatase Gene. In four different strains (wild type, PS I-less, PS I-less/*psb*DIC$^-$/*psb*DII$^-$, and PS I-less/*psb*B$^-$/*psb*C$^-$/*apc*E$^-$) (*11*, *13*, *18*) the ferrochelatase (*scp*A) gene was interrupted at the *Bsp*1286I site in the gene by insertion of a 1.6 kb kanamycin-resistance marker. This restriction site corresponds in the protein to the region connecting the ferrochelatase and SCP domains. Insertion of the kanamycin-resistance marker led to a stop of the ferrochelatase gene 45 bp beyond the site of insertion. The amino acid sequence that is added to ferrochelatase is MDSLNDPPCTFETVP. The remaining 163 bp of the *scp*A part of the ferrochelatase gene are not expressed. Transformants were allowed to segregate at moderate (50 μE m^{-2} s^{-1}; wild type) or low (5 μE m^{-2} s^{-1}; PS I-less strains) light intensity.

Southern Hybridization. To make sure that homozygosity had been attained at the *scp* loci, we performed Southern hybridization with genomic DNA from the different transformants using wild-type DNA as template for PCR with radiolabeled dATP (*11*). DNA of the segregated mutants was sequenced to confirm the desired mutation.

Thylakoid Preparations and Protein Blotting. Thylakoids from different *Synechocystis* 6803 mutants were prepared as described (*16*). Peptide-directed antibodies against an N-terminal region of ScpC and ScpD (TRGFRLDQDN) were made commercially and used to identify these polypeptides after separation of thylakoid proteins via high-resolution SDS−PAGE as has been described (*19*).

Chlorophyll Analysis. Chlorophyll *a* was extracted from cells with 100% methanol, and concentrations were determined according to ref *20* using an UV-160 spectrophotometer.

Absorption, Fluorescence Emission, and Oxygen Evolution. Room-temperature absorption spectra from 400 to 800 nm were measured on a Shimadzu UV-160 spectrophotometer.

Fluorescence emission spectra (77 K) were recorded on a Perkin-Elmer luminescence spectrometer. The cells were frozen in BG-11 plus 20 mM HEPES/NaOH (pH 7.5) without glycerol to prevent disruption of the phycobilisomes. Excitation was performed at 440 nm for chlorophyll excitation or 590 nm for phycobilin excitation. Each sample had a chlorophyll concentration of 5 μg mL^{-1}. Spectra were corrected for wavelength-dependent sensitivity of the photodetector.

O_2 evolution was detected with a Clark-type electrode. Cells were harvested at OD$_{730}$ ~0.7−0.8, washed once in BG-11 medium supplemented with 20 mM HEPES/NaOH (pH 7.5), and then suspended at a concentration of 10 μg mL^{-1} chlorophyll in the same buffer. Measurements were performed at 25 °C in the presence of 0.5 mM $K_3Fe(CN)_6$ and 0.1 mM 2,6-dichloro-*p*-benzoquinone (DCBQ) with yellow actinic light (570 nm cutoff filter) at a saturating intensity of 3000 μE m^{-2} s^{-1}, or with light passed through a 600 nm broad-band interference filter (phycobilisome excitation) or through a red cutoff filter ($\geq$665 nm) (mostly chlorophyll excitation).

RESULTS AND DISCUSSION

Synechocystis 6803 Genes with Similarity to the Cab Family. A computer search for genes coding for Cab-like proteins in the genome of *Synechocystis* 6803 (see CyanoBase at http://www.kazusa.or.jp/cyano/cyano.html) identified

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             1
                                                                 50
                                                     *    ** *  **       *      *
ELIP (I)                                FSDLM AFSGPAPERI NGRLAMIGFA AMGV.EIAKG QGL
ScpC         MTTRGFRLDQD NRLNNFAIEP EVYVDSSVQA .GWTKYAEKM NGRFAMIGFA SLLIMEVVTG HGVIGWLNS L..
ScpD         MTSRGFRLDQD NRLNNFAIEP PVYVDSSVQA .GWTEYAEKM NGRFAMIGFV SLLAMEVITG HGIVGWLNS L..
HliA         .MRSGRTVDEF GRQNNFAIEP QVVVAEADAS WGFHDRAEKL NGRLAMIGFV ALILTEVALG QGLLPFLAS GLS
ScpB         .MGAILCYIYL HRQPSQLVIT FLTMNNENSK FGFTAFAENW NGRLAMIGFS SALILELVSG QGVLHFFGI L..
ScpE         ........... ...MSEELQP NQTPVQEDPK FGFNNYAEKL NGRAAMVGFL LILVIEYFTN QGVLAWLGL R..
ScpA         .MDSLNDPPCT FETVPHPKKN MKMYPQERWE WGLTTAAEVW NGRLAMLGFI ALLV.ELISG QGPLHFVGL L..
PSII-S (III)                            EGGPL FGFTKANELF VGRLAQLGFA FSLIGEIITG KG
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FIGURE 1: Sequence alignment of the five small Cab-like proteins (SCPs) of *Synechocystis* 6803, the *hliA* gene product of *Synechococcus* 7942, and the first or third membrane-spanning helices of the Cab family from higher plants (ELIPs from pea and PS II−S from spinach). Note that ScpA is a polypeptide expected to have both ferrochelatase- and Cab-like characteristics, and only the C-terminal region of the polypeptide (starting at amino acid residue 319) has been shown.

five different genes. The translated sequences of these genes have a high degree of similarity to each other, to HliA of *Synechococcus* 7942, and to the third helix of Cab proteins (Figure 1); the similarity to the first helix of Cab proteins is somewhat lower. The predicted products of the five open reading frames are generally small (6−8 kDa) and have been named small Cab-like proteins (SCP). The five genes for SCPs appear to form a multigene family. The presence of such families in prokaryotes used to be viewed as uncommon, but with the sequencing of entire prokaryotic genomes, a number of examples of multigene families have now become apparent (*21*, *22*). The five open reading frames are scattered throughout the cyanobacterial genome, and their locations have been listed in the Materials and Methods section. However, one of these predicted *Synechocystis* 6803 polypeptide sequences with similarity to Cab proteins, ScpA, was found to be larger (387 residues). Only the C-terminal part of ScpA showed similarity to Cab proteins; the remainder of the protein (the N-terminal part up to 69 residues from the C-terminus) had strong similarity to ferrochelatase from other organisms.

As shown in Figure 1, the other four predicted *Synechocystis* 6803 polypeptides with similarity to the first and third transmembrane regions of Cab proteins were small and ranged in size from 57 to 70 amino acid residues. They have been named ScpB, ScpC, ScpD, and ScpE. ScpC and ScpD were found to be very similar to each other (see Figure 1). The C-terminal 69 residues of ScpA (the nontypical ferrochelatase domain) are most similar to the PS II−S protein of higher plants (*3*, *4*). ScpB most resembles the ELIP from pea (*23*), and ScpC, ScpD, and ScpE have the highest similarity to HliA from *Synechococcus* 7942 (*10*). The HliA, ELIPs, and PS II−S proteins may function in different stresses, mainly in light stress (*24*). ELIPs as well as the PS II−S protein have been suggested to act as chlorophyll scavengers upon formation and breakdown of chlorophyll-binding proteins (*25*, *26*). Both, ELIPs and the PS II−S protein have been proposed to bind xanthophyll-cycle pigments in intermittent light-grown plants, which do not accumulate chlorophyll *a*/*b* proteins and may act as sinks for excitation energy at high light intensity (*27*, *28*).

Chlorophyll- and carotenoid-binding regions of Cab proteins have been conserved in the SCPs, suggesting that SCPs indeed may bind pigments. The residues in the NGRLAMIGF consensus region (residues 41−49 in Figure 1), where in Cab proteins the transmembrane helices 1 and 3 cross each other and where carotenoids are located (*1*), are highly conserved. In the LHCII structure the positively charged guanidinium group of the arginine side chain (residue 43) is placed in juxtaposition to the negatively charged glutamic acid side chain (homologous to that of residue 38 in Figure 1) from the other helix. These residues that together bind chlorophyll in LHCII (*1*) are present in all five SCPs and are conserved in HliA as well (*10*). The chlorophyll-binding residue His68 of LHCII is replaced by Asn in SCPs, HliA, and ELIPs (residue 41 in Figure 1). Asn generally can functionally substitute for chlorophyll-binding His residues (see ref *18*) and again would be compatible with pigment binding to SCPs. Moreover, a Glu (residue 56) can be found in all SCPs and also in the PS II−S protein and in the antenna protein CP24 (*29*); it corresponds to a chlorophyll-binding Gln in LHCII (*1*). Other conserved residues not directly related to pigment binding include two glycines (residues 32 and 62 in Figure 1) that, according to the LHCII structure, are close to either end of the membrane-spanning helix.

Ferrochelatase. The open reading frame encoding ScpA is 1161 bp in length, of which the 5′ 80% has striking similarity to ferrochelatase genes from other organisms. The 3′ 207 bp resemble the other four *scp* genes. DNA sequencing of the junction region between the ferrochelatase and SCP gene regions confirmed that the ORF codes for a joint ferrochelatase/SCP polypeptide (data not shown). Therefore, there is no frame shift or stop codon that had been missed in the genomic sequencing project. No other obvious ferrochelatase gene is apparent in the *Synechocystis* 6803 genomic sequence. The phenomenon of merging two seemingly unrelated functions into a single open reading frame also has been observed in the case of the *cpc*D and *pet*H genes (*30*), which code for a phycobilisome component and the ferredoxin−NADP$^+$ oxidoreductase, respectively. Interestingly, the same ferrochelatase/SCP gene connection can be found in one of the two *Arabidopsis thaliana* ferrochelatase isoforms known to date (ferrochelatase 2; ref *31*) and in rice (*Oryza sativa*; ref *32*) (Figure 2). Ferrochelatase 2 of *Arabidopsis thaliana* is exclusively plastidic, whereas ferrochelatase 1 appears to be targeted to both chloroplasts and mitochondria. However, an SCP-like extension is absent from ferrochelatases from all nonphotosynthetic organisms (including yeast, mammals, and bacteria).

To provide information regarding the function of ScpA, we introduced a kanamycin-resistance marker at the *Bsp*1286I

Hv	FISDLADAVV EALP..... ....SA SAMATRKV.. KDTDSD.MDM MHYLTKMFLG SVLAFFLLLS PRLVSAFRNT LQ*...... ....
Cs	FISDLADAVI EALP..... ....SA TALAPHTS.. .STDADDHDP FLYAIKLLFG SVLAFILLLS PKAFMVFRNN FLLNYTRIYG YRGERSEFFW VRLIFT*
At 1	FITDLADAVI ESLP..... ....SA EAMSNPNAVV DSEDSESSDA FSYIVKMFFG SILAFVLLLS PKMFHAFRNL*......... ....
Os	FITDLADAVI ESLPYVGAMA VSNLEARQPL VPLGSVEELL AAYDSKRDEL PPPVTVWEWG WTKSAETWNG RAAMLAVLAL LVLEVTTGEG FLHQWGILPLFH*...
At 2	FISDLADAVV ESLPYVGAMA VSNLEARQSL VPLGSVEELL ATYDSQRREL PAPVTMWEWG WTKSAETWNG RAAMLAVLAL LVLEVTTGKG FLHQWGILPS L*....
ScpA	FIDALAQMV**M DSLN..... ....DPPCTFE TVPHPKKNMK MYPQERWEWG LTTAAEVWNG RLAMLGFIAL LV.ELISGQG PLHFVGLL***. ......
Hs	FSKALADLVH SHIQSNELCS KQLTLSCPLC .VN.PVCRET KSFFTSQQL*.. ....
Mm	FSKALADLVH SHIQSNKLCS TQLSLNCPLC .VN.PVCRKT KSFFTSQQL*.. ....
Sc	FIEGMADLVK SHLQSNQLYS NQLPLDFALG KSNDPVKDLS LVFGNHEST* ....
Bs	FIDALATVVL KKLGR*.... ....
Bj	GMDVIRTLVL RELQGWI*.. ....

FIGURE 2: Sequence comparison of ferrochelatases from barley (*Hv*), cucumber (*Cs*), *Arabidopsis thaliana* (*At*), rice (*Os*), *Synechocystis* 6803 (*scp*A), human (*Hs*), mouse (*Mm*), yeast (*Sc*), *Bacillus subtilis* (*Bs*), and *Bradyrhizobium japonicum* (*Bj*). The sequence alignment shown starts at amino acid residue 401 of barley ferrochelatase. The diverging *Synechocystis* 6803 sequence that has homology to Cab-like proteins has been bolded. Of the 387 amino acids of ferrochelatase from *Synechocystis* 6803, the first 318 amino acids have 55% identity to barley, 53% to cucumber, 54% to *Arabidopsis*, 37% to yeast, 40% to mouse, and 39% to human ferrochelatase. The whole *Synechocystis* ferrochelatase has 60% identity (67.8% similarity) to the rice ferrochelatase and 61.5% identity (68.5% similarity) to the second ferrochelatase of *Arabidopsis thaliana*.

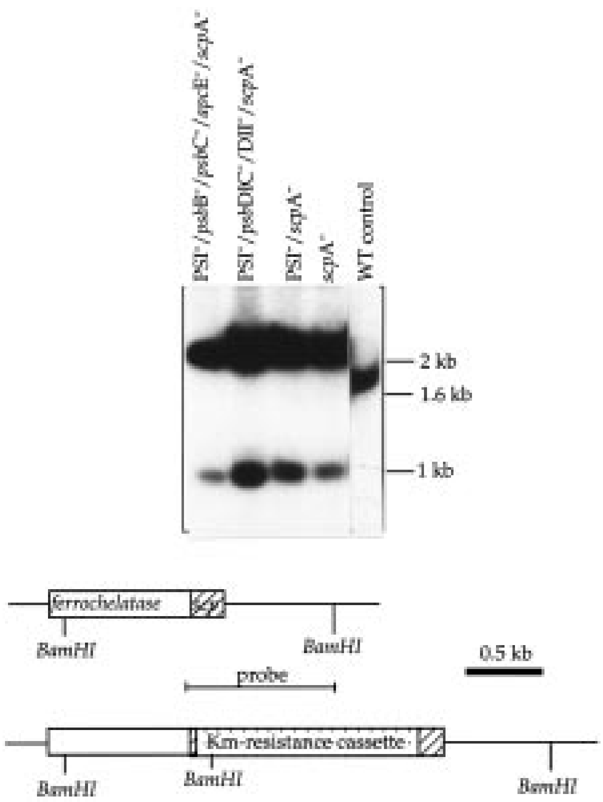

FIGURE 3: Southern hybridization of *scp*A interruption mutants in wild type, the PS I-less strain, the PS I-less/*psb*DIC$^-$/*psb*DII$^-$ strain, and the PS I-less/*psb*B$^-$/*psb*C$^-$/*apc*E$^-$ strain. As a control, genomic DNA of nontransformed wildtype was used. The restriction enzyme used for genomic DNA digestion was *Bam*HI; the size of the wild-type DNA recognized by the probe was 1.8 kbp, and insertion of the kanamycin-resistance marker in *scp*A added 1.2 kbp to this size.

restriction site that spans the codons V332 and P333 of *scp*A, which are at the border of the 5′ 954 bp ferrochelatase domain and the 3′ 207 bp Scp extension of *scp*A (Figure 3). In the interruption mutant, ferrochelatase activity is expected to remain but the C-terminal extension with its Cab-like domain will be absent from the truncated ferrochelatase protein. In the determination of whether segregation of wild-type and mutant genotypes with respect to the *scp*A locus had occurred, DNA isolated from different *scp*A transformant strains was digested with *Bam*HI restriction endonuclease and used for Southern hybridization. The Southern blot (Figure 3) shows segregation of *scp*A mutants in four different backgrounds: wild type, the PS I-less strain, the PS I-less/PS II-less strain, and the PS I-less/*psb*B$^-$/*psb*C$^-$/*apc*E$^-$ strain.

Even though the *scp*A gene interruption was introduced such that ferrochelatase should remain active, a modification of ferrochelatase activity due to an altered lifetime, docking, and/or activity of the truncated protein could not be excluded. Reduced ferrochelatase activity in the *scp*A interruption mutants might lead to decreased heme and phycobilin levels. To determine whether there is evidence for changes in phycobilin levels, we measured absorption and 77K fluorescence emission spectra of the PS I-less/*scp*A interruption mutant and a control PS I-less strain. Pigmentation in the mutant was essentially identical to that of the control with the ratio of the peak at around 625 nm (due to phycocyanin) to that at around 670 nm (due mostly to chlorophyll) indistinguishable between the two strains (data not shown). In addition, 77K fluorescence emission spectra were unaltered (data not shown). The presence of ferrochelatase in the *scp*A mutant could not be quantitated directly as antibodies raised against ferrochelatases from other organisms did not crossreact with the cyanobacterial protein and peptide-directed antibodies against the N-terminus of *Synechocystis* 6803 ScpA were found to be not antigenic.

The interruption of *scp*A in the *Synechocystis* 6803 wild-type strain did not alter photoautotrophic growth under different light conditions (not shown). However, an interesting phenotypic consequence of *scp*A interruption in the PS I-less background strain was that the resulting mutant had an increased light tolerance. When grown at moderate light intensity (50 μE m^{-2} s^{-1}) for two weeks, the PS I-less strain was bleached out entirely, whereas the PS I-less/*scp*A$^-$ mutant retained significant pigmentation (*33*). Based on this, the light tolerance of the PS I-less strain appeared to have increased at least 5-fold upon interruption of the ferrochelatase gene. This phenotype was observed in six independently generated *scp*A$^-$ mutants. This increase in light

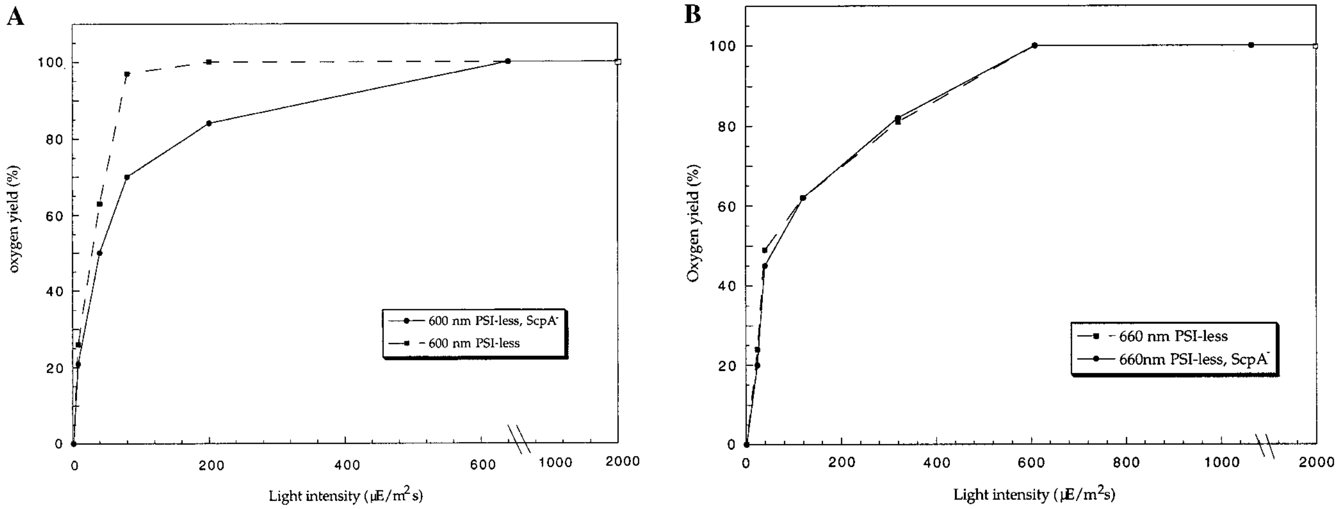

FIGURE 4: Light intensity dependence of oxygen evolution rates. Oxygen evolution was measured as a function of light intensity in PS I-less strains with and without ScpA with (A) light passed through a 600 nm broad-band interference filter and (B) light passed through a red cutoff filter transmitting light of wavelengths ≥665 nm.

Table 1: Amount of Total RNA in Each Northern Blot Lane in Comparison with the Probe Hybridization Density Measured by Phosphoimager[a]

strain	light intensity	loading (μg of total RNA)	transcript accumulation (a.u.)					
			*rps*1	*scp*A	*scp*B	*scp*C	*scp*D	*scp*E
wild type	HL	33	211	215	48	1	162	28
	NL	17	270	233	33	11	60	7
	LL	5.4	329	238	42	19	100	39
	LAHG	3.6	369	224	40	46	172	23
PS I-less	LL	8.1	270	206	94	199	332	62
	LAHG	9.0	208	204	109	171	314	66
PS II-less	NL	21	178	215	23	0	90	6
	LL	8.2	290	210	50	41	150	44
	LAHG	6.7	145	187	36	41	105	38
PS I-less/PS II-less	NL	19	252	203	114	257	284	85
	LL	19	320	207	161	318	480	153
	LAHG	41	182	210	101	124	216	95
PS I-less/*chl*L$^-$	LL	24	231	223	54	132	257	25
	LAHG	92	109	190	10	28	118	9

[a] Data on transcript accumulation come from the blots shown in Figure 5.

tolerance of the PS I-less strain might be caused by either a decreased light-harvesting efficiency of the *scp*A$^-$ mutant, or a decrease in the presence of photooxidative compounds such as free chlorophyll.

To distinguish between these possibilities, we determined the light-harvesting efficiency in the PS I-less wild type and *scp*A$^-$ strains. The light intensity at 600 nm (phycobilisome absorption) that was needed for oxygen evolution at a rate half of that at saturating light intensity was increased by about 25%, whereas no significant difference was found in the efficiency of utilization of light absorbed by chlorophyll (665 nm) (Figure 4). These results suggest that the light energy transfer efficiency from phycobilisomes to PS II is decreased somewhat upon *scp*A interruption (corroborating the concept that *scp*A binds chlorophyll), but this decrease is insufficient to account for the large increase in light tolerance. Therefore, it is attractive to speculate that the *scp*A interruption might decrease the rate or capacity of chlorophyll synthesis and thereby minimize the amount of free chlorophyll or related pigments in the membrane.

The question is how *scp*A interruption might affect the accumulation of photooxidative chlorophyll or one of its precursors. Heme and chlorophyll biosynthesis pathways compete for a common substrate (protoporphyrin IX), and increasing ferrochelatase activity will provide fewer resources for chlorophyll production and vice versa. The SCP domain in ferrochelatase may serve a regulatory function with the enzyme most active if this domain contains chlorophyll or is absent, thus increasing the probability of Fe vs Mg chelation of protoporphyrin IX. Conversely, in the absence of Fe more chlorophyll may be formed. In this context it is important to note that, upon iron starvation, cyanobacteria produce a chlorophyll-binding protein encoded by *isi*A; this protein is related to the PS II antenna protein CP43 and may act as a chlorophyll reservoir to be used upon recovery from iron limitation (*34*).

Transcription of scp Genes. The *hli*A gene in *Synechococcus* 7942 was reported to be transcribed mainly under high light conditions (*10*) and is similar to members of the *scp* gene family of *Synechocystis* 6803. Therefore, transcript analysis under different growth conditions was performed to provide information regarding the expression of these genes as a function of light intensity. Figure 5A shows Northern blots of RNA from wild-type *Synechocystis* 6803 as well as from PS II-less, PS I-less/PS II-less, and PS I-less/*chl*L$^-$ strains grown at different light intensities and probed with *scp*A, *scp*B, *scp*C, *scp*D and *scp*E. Light intensities were high (250 μE m^{-2} s^{-1}), normal (50 μE m^{-2} s^{-1}), low (5 μE

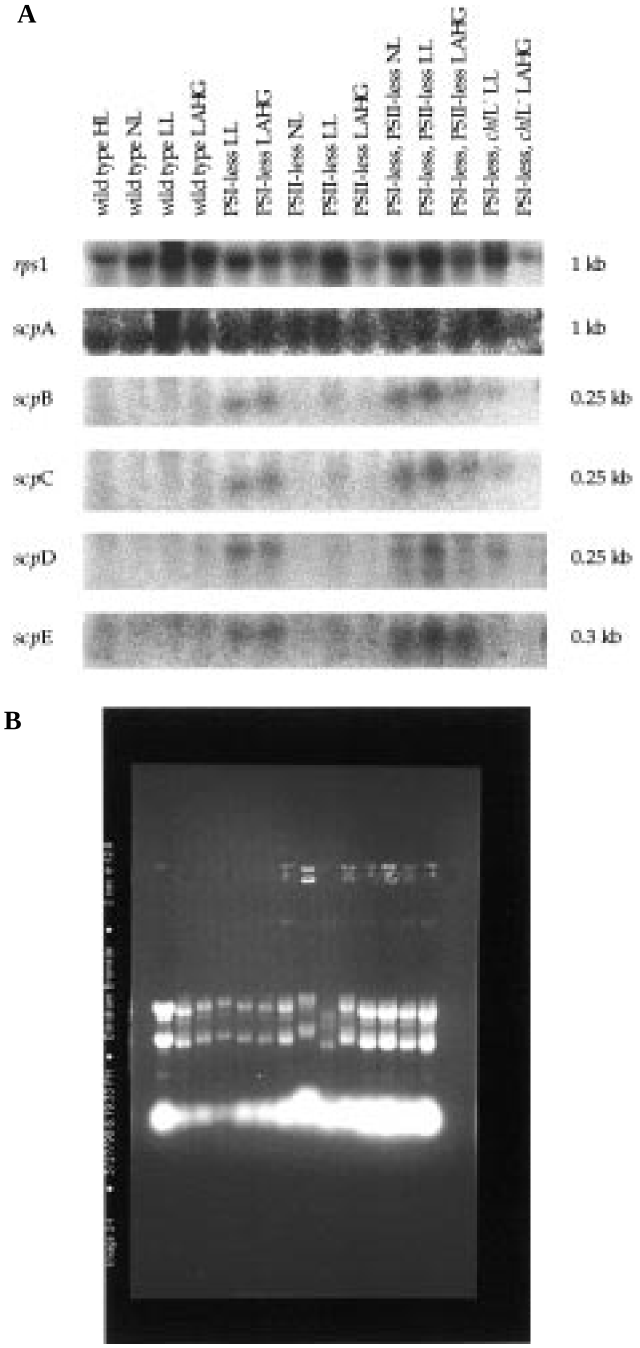

FIGURE 5: Transcript levels of *scp* genes. (A) Northern hybridization of RNA isolated from *Synechocystis* 6803 wild type and the PS I-less, PS II-less, PS I-less/PS II-less, and PS I-less/*chl*L$^-$ strains grown at high (HL), normal (NL), and low light (LL) intensities (250, 50, and 5 μE m^{-2} s^{-1}, respectively) and under light-activated heterotrophic growth (LAHG) conditions and probed with *scp*A, *scp*B, *scp*C, *scp*D, and *scp*E. As a control, northern hybridization with *rps*1 was performed. (B) Ethidium bromide-stained RNA gel in which the same amount of RNA has been loaded as in panel A.

m^{-2} s^{-1}), or essentially 0 (light-activated heterotrophic growth (LAHG) conditions, ref *35*). To be able to compare relative levels of mRNA (as opposed to total RNA) in the different conditions, we probed Northern blots with the *rps*1 gene as an internal standard. The amount of rRNA in each sample is shown in Figure 5B. Table 1 provides the amount of total RNA loaded in each lane in relation to the density of Northern blot signals measured by a phosphorimager. It is clear that the ratio of the amount of RNA and the amount of *rps*1 transcript under various conditions and in different strains varies significantly, indicating that the entity to which transcript levels should be normalized is difficult to establish. For this reason, in our study, *rps*1 and rRNA as well as total RNA levels have been presented. As may be expected, the amount of *rps*1 transcript relative to the amount of total RNA generally is highest under relatively unstressed conditions (moderate light or LAHG conditions).

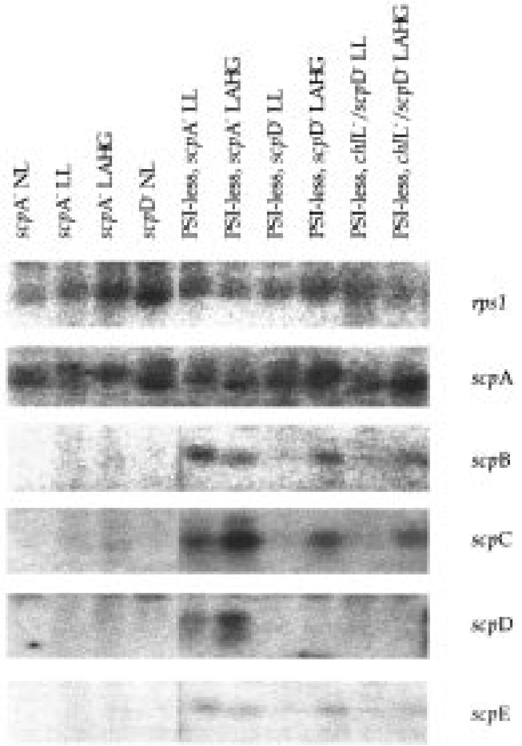

FIGURE 6: Northern hybridization of RNA isolated from *Synechocystis* 6803 mutants with interrupted *scp*A or *scp*D grown under normal (NL) or low light (LL) intensity (50 and 250 μE m^{-2} s^{-1}, respectively) or under light-activated heterotrophic growth (LAHG) conditions and probed with *scp*A, *scp*B, *scp*C, *scp*D, and *scp*E. As a control, northern hybridization with *rps*1 was performed.

Transcript sizes were 1 kb for *scp*A, 0.25 kb for *scp*B, *scp*C, and *scp*D, and 0.3 kb for *scp*E, suggesting monocistronic transcription of these genes. The transcription levels, which were rather similar for *rps*1 for the loadings used, were also similar for *scp*A in the different mutants grown under different light conditions. For the other four *scp* genes the presence or absence of PS I had a large effect on transcript accumulation. In wild-type cells and PS II-less cells little *scp*B, *scp*C, *scp*D, and *scp*E transcript accumulated, whereas transcript accumulation was increased in the PS I-less, PS I-less/PS II-less, and, to a lesser extent, also in the PS I-less/*chl*L$^-$ strains. In particular, expression of *scp*C and *scp*D was much induced upon the removal of PS I.

PS I-less mutants are light-sensitive and can be easily light-stressed, but under the low light conditions used for the PS I-less strains no light stress occurs. Indeed, even under LAHG conditions high *scp* transcript and protein levels can be detected in PS I-less strains. This, coupled to the fact that the level of *SCP* transcripts in wild type is modest, even at high light intensity, indicates that the SCPs are not light-stress proteins.

As PS I in cyanobacteria binds most of the chlorophyll present in the cell, we suggest that the increased expression

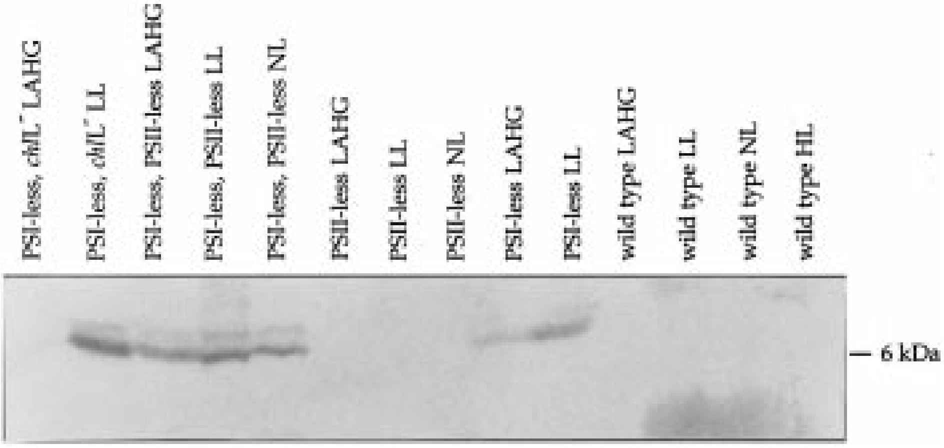

FIGURE 7: Immunodetection of ScpC and ScpD in thylakoids isolated from *Synechocystis* 6803 wild type and PS I-less, PS II-less, PS I-less/PS II-less, and PS I-less/*chl*L$^-$ mutant strains grown under normal light (NL; 50 μE m^{-2} s^{-1}), low light (LL; 5 μE m^{-2} s^{-1}), and/or light-activated heterotrophic growth (LAHG) conditions with peptide-directed antibodies. Loading was done on a chlorophyll basis, taking into account the large differences of the amounts of chlorophyll per cell. The total amount of protein in each lane was similar. Amount of chlorophyll loaded per lane: wild type, 3 μg; PS I-less strain, 0.6 μg; PS II-less strain, 2.4 μg; PS I-less/PS II-less strain, 0.15 μg; and the PS I-less/*chl*L$^-$ strain grown under LAHG conditions for 2 days, 0.15 μg.

of the four small SCPs is a consequence of the lower requirement for chlorophyll by the cell. SCPs may function as low-affinity chlorophyll-binding proteins that eliminate the presence of free chlorophyll in thylakoid membranes: they may bind chlorophyll as it is available and transfer it to reaction centers and antenna as they are synthesized. The presence of chlorophyll in the PS I/PS II mutant (*18*) indicates that some chlorophyll is bound to proteins that are not associated with the reaction centers. The four small SCPs might be associated with such chlorophyll and function to prevent possible photooxidative damage and/or to serve as peripheral antenna. In this light, the observation of the induction of *hli*A at high light intensity (*10*) may be explained to provide a binding environment for chlorophyll as reaction centers are damaged and degraded.

The discussion above implies that ScpA is functionally different from the other four small SCPs. Therefore, if this is correct no compensatory effects on transcript levels are expected upon interruption of *scp*A vs inactivation of one of the other *scp* genes. In Figure 6 Northern blots are shown for RNA from the *scp*A interruption mutant grown under different light conditions and also from a *scp*D interruption mutant in the PS I-less background. Interestingly, for none of the *scp* genes are transcript levels greatly affected upon alteration of *scp*A or *scp*D, indicating that little compensation of transcript levels occurs for any of the *scp* genes. Note that transcripts of *scp*A are detectable also in the *scp*A interruption mutant, as the primers used for probe preparation by PCR were chosen to amplify DNA 5′ of the site interrupted by the kanamycin-resistance cartridge. As single mutants lacking *scp*B, *scp*C, *scp*D, or *scp*E have a relatively normal phenotype (Hong Xu and Wim Vermaas, unpublished), a study of other *scp* mutants has not been part of this project.

Translation of the scp Genes. Synthesis and accummulation of Cab proteins are not correlated to the transcript levels in plants (*37*). To investigate whether the increased transcript levels in the PS I-less strains are reflected in increased levels of corresponding proteins, we raised peptide-directed antibodies against the N-termini of ScpC and ScpD, which are very similar. Thylakoid proteins isolated from the different mutants grown at various light conditions were probed with these antibodies after protein separation by Tricine/SDS–PAGE (*36*). As indicated in Figure 7, ScpC and/or ScpD run as polypeptides of about 6 kDa, and their accumulation seems to follow the trends that are apparent in transcript accumulation: the strongest immunoresponse with polypeptides in the 6 kDa region was detected in mutants lacking PS I. A weak immunodecoration was also visible in wild-type cells grown under high light conditions (250 μE m^{-2} s^{-1} for 12 h). Under conditions of increased light stress (1000 μE m^{-2} s^{-1} for 4 h), a stronger immunoresponse with the ScpC/ScpD antibody was detected (not shown), but the level of ScpC/ScpD in wild type was never found to approach the amount detected in mutants lacking PS I. In these strains, the amount of chlorophyll per cell is low: deletion of PS I leads to a 6-fold decrease in the amount of chlorophyll per cell (*17*), whereas PS II deletion does not significantly affect this. Therefore, ScpC/ScpD accumulation may be linked to a low chlorophyll requirement of cells, and these proteins may function as a chlorophyll-binding buffer to absorb excess chlorophyll that is being synthesized.

It is unlikely that the redox state of thylakoid components significantly affects *scp* expression. In the PS II-less/PS I-less strain, in which the quinone pool is expected to be rather oxidized under aerobic conditions, expression was rather similar to that in the strain lacking PS I only, in which the quinone pool is expected to be rather reduced in the light.

To distinguish whether immuno reaction occurred mostly with ScpC or ScpD, immuno blot using thylakoids from the PS I-less/*scp*D$^-$ mutant was compared with that using PS I-less and PS II-less thylakoids (Figure 8). In the PS I-less/*scp*D$^-$ mutant only a faint band at a slightly lower molecular weight could be observed after immunodecoration in comparison to the PS I-less mutant. We suggest that the lower band is due to ScpC, which may accumulate in slightly larger amounts in the absence of ScpD. Therefore, ScpD is more prominent in thylakoids of PS I-less strains than ScpC.

Interestingly, a band with a molecular weight of more than 45 kDa was detected that was absent in thylakoids of the PS I-less/*scp*D$^-$ strain. This indicates that this band contains ScpD and suggests that this may be an aggregation product

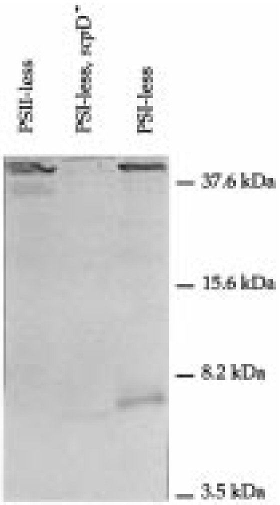

FIGURE 8: Immunodetection of ScpC and ScpD in thylakoids from the PS II-less, PS I-less/*scp*D$^-$, and PS I-less strains. Amounts of chlorophyll loaded per lane were identical to those in Figure 5. Note the presence of a high molecular weight band (above 45 kDa) that disappears in the PS I-less/*scp*D$^-$ strain.

that remains stable in denaturing SDS/PAGE. This aggregation product may contain proteins other than SCPs but may also be composed of stable SCP multimers (for example, a multimer). In this context it is important to realize that pigment binding in LHCII requires the close interaction between the first and third transmembrane helices. Therefore, SCPs may need to form multimers with an even number of helices in order to bind pigments.

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