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The small CAB-like proteins of the cyanobacterium *Synechocystis* sp. PCC 6803: Their involvement in chlorophyll biogenesis for Photosystem II

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ABSTRACT

The five small CAB-like proteins (ScpA-E) of the cyanobacterium *Synechocystis* sp. PCC 6803 belong to the family of stress-induced light-harvesting-like proteins, but are constitutively expressed in a mutant deficient of Photosystem I (PSI). Using absorption, fluorescence and thermoluminescence measurements this PSI-less strain was compared with a mutant, in which all SCPs were additionally deleted. Depletion of SCPs led to structural rearrangements in Photosystem II (PSII): less photosystems were assembled; and in these, the Q_B site was modified. Despite the lower amount of PSII, the SCP-deficient cells contained the same amount of phycobilisomes (PBS) as the control. Although the excess PBS were functionally disconnected, their fluorescence was quenched under high irradiance by the activated Orange Carotenoid Protein (OCP). Additionally the amount of OCP, but not of the iron-stress induced protein (isiA), was higher in this SCP-depleted mutant compared with the control. As previously described, the lack of SCPs affects the chlorophyll biosynthesis (Vavilin, D., Brune, D. C., Vermaas, W. (2005) Biochim Biophys Acta 1708, 91–101). We demonstrate that chlorophyll synthesis is required for efficient PSII repair and that it is partly impaired in the absence of SCPs. At the same time, the amount of chlorophyll also seems to influence the expression of ScpC and ScpD.

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1. Introduction

During evolution, photosynthetic organisms have adopted and retained different types of antenna pigment–protein complexes. In plants, the most abundant peripheral antennae are the intrinsic chlorophyll *a*/*b*-binding light-harvesting complexes, encoded by a multi-gene family of at least 10 different *cab* genes [1]. All CAB proteins in plants are related to each other, according to their protein sequence; they all contain the chlorophyll-binding CAB domain, and are assumed to share a common evolutionary origin [2,3]. The nuclear genes code for light-harvesting chlorophyll proteins with three membrane-spanning regions per polypeptide [4,5].

However, the family also includes genes coding for light-harvesting-like (Lil) proteins, that instead of harvesting light energy are thought to be involved in photoprotection [6]. The large family of Lil proteins in plants consists of proteins predicted to have four membrane spanning helices (PsbS, [7,8]), three transmembrane α-helices (early light induced proteins: ELIPs, [9]), two α-helices (stress enhanced proteins: SEPs, [10]), and proteins with a single membrane span, referred to as one helix proteins (OHPs), high-light induced proteins, (HLIPs), or small CAB-like proteins (SCPs) [3,11]. Lil proteins are regulated opposite to the light-harvesting proteins; under high light conditions—when the expression of the LHC proteins is repressed—Lil proteins are up-regulated. This indicates a function in high-light protection in a broad sense; they might provide direct protection (as shown for PsbS, [12]), have an effect on pigment stability and/or act as pigment carriers [13–16], or have novel functions, not yet detected. It is likely that they bind pigments (chlorophylls and carotenoids) [17–19], although indisputable evidence for this is lacking.

Instead of the multi-helix CAB proteins, the major peripheral light-harvesting complex in cyanobacteria is the extrinsic phycobilisome (PBS). Still, cyanobacteria contain single-helix Lil proteins [20,21], which are thought to be the evolutionary ancestors of eukaryotic CAB proteins [6]. The conserved structure of the Lil proteins and light-harvesting complexes among oxygenic photosynthetic organisms implicates their involvement in essential processes. The important

Abbreviations: CAB, chlorophyll *a*/*b* binding; DCMU, 3-(3, 4-dichloro-phenyl)-1, 1-dimethylurea; Lil, light-harvesting-like proteins; OCP, orange carotenoid protein; PS, Photosystem; SCP, small CAB-like proteins

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function of these proteins is evident in the finding that the much reduced genome of the high-light ecotype *Prochlorococcus marinus* strain Med4, belonging to a sub-group of cyanobacteria, encodes at least 24 *scp*-genes [20]. Relatives to the SCPs have even been identified in cyanophages of marine cyanobacteria [22,23]. In the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter: *Synechocystis* 6803), five small Lil proteins were identified with similarity to the first and third helix of the CAB-proteins [21]. These small CAB-like proteins (SCPs) are induced not only under high light—leading to their name high-light inducible proteins (HLIPs), but also during nutrition or temperature stress [21,24]. Additionally, their genes were found to be induced in mutants lacking Photosystem I (PSI) [21]. Four of them (*scp*B-E) encode proteins of around 6 kDa, while the fifth one (*scp*A) is part of the ferrochelatase gene (*hem*H) and encodes the C-terminal extension of the ferrochelatase [21]. Their importance in stabilizing chlorophyll-binding proteins is well documented [15,16,25–27], they might also be important for the tetrapyrrole metabolic pathway [15,16,28] or act as pigment-carriers [27,29].

ScpB-E are located close to Photosystem II (PSII) [29–31]. While inactivation of single *scp* genes leads to no obvious phenotype [21], multiple *scp* deletion in *Synechocystis* 6803 altered cell pigmentation [14] and reduced the amount of chlorophyll, carotenoids, and phycobilisomes [15,32]. A mutant with ScpB-E (*scp*BCDE or *hli*ABCD) inactivated was not able to survive high-intensity illumination and showed alteration in the ability to perform non-photochemical dissipation of absorbed light energy [32]. The phenotype observed upon deletion of *scp* genes was enhanced in mutants lacking PSI [15]. The PSI-less/ScpABCDE^{-} mutant only contained one third of the chlorophyll amount of a PSI-less control [15]. Moreover, the half-life of chlorophyll molecules was shortened 5-fold [27] in that strain. It therefore was concluded that the SCPs prevent degradation of PSII-associated chlorophyll, but do not alter the lifetime of chlorophyll associated with PSI [27]. Here we perform a detailed comparison of a PSI-less control strain with the PSI-less/ScpABCDE^{-} strain to elucidate the structural and functional role of SCPs within PSII. We observed that SCPs are not involved in cyanobacterial non-photochemical quenching (NPQ), however, the strain lacking the five SCPs has a lower PSII/PBS ratio than the control strain, in agreement with the functions proposed for SCPs as mediators of PSII stabilization and/or repair under stress conditions. This finding was reinforced by a detailed analysis of PSII biophysical properties, which showed that although the PSII centers of the control strain and the mutant are able to perform charge separation at similar rates, the Q_B site seems to be unstable in the absence of the SCPs. In addition, the amount of Photosystem II is decreased in this mutant. Furthermore, photoinhibition was enhanced in the absence of SCPs. Interesting also was the observation that in the presence of gabaculine, an inhibitor of chlorophyll synthesis, the Photosystem II recovery after induced damage was reduced in the SCP-less mutant strain.

2. Materials and methods

2.1. Strains and growth conditions

Glucose-tolerant PSI-less [33] and PSI-less/ScpABCDE^{-}[15] strains of *Synechocystis* 6803 were grown at 30 °C in liquid BG-11 medium [34] supplemented with 10 mM glucose at a light intensity of 2–4 μmol photons m^{-2} s^{-1}. Liquid cultures in their exponential phase (OD_{730} 0.4–0.8), were harvested and used for the different measurements.

2.2. Low temperature fluorescence spectroscopy

Low temperature (77 K) fluorescence emission spectra were determined using a FluoroMax-2 fluorometer (Spex spectrofluorometer system, Jobin Yvon, Longjumeau, France) with excitation and emission slit widths of 1 and 0.5 mm, respectively. Whole cells were concentrated to 2 μg/mL chlorophyll in BG11 without glycerol added. The maximum peak of the different spectra was normalized to 1.

2.3. Room temperature fluorescence

The yield of chlorophyll fluorescence was monitored in a modulated fluorometer (PAM; Walz, Effelrich, Germany). NPQ induction and recovery experiments were performed in a stirred cuvette of 1 cm diameter (32 °C) at a chlorophyll concentration of 0.4 μg/mL in BG11. When indicated 5 μM DCMU was included.

2.4. Fluorescence relaxation kinetics

Flash-induced increase and subsequent decay of chlorophyll fluorescence yield was measured by a double-modulation fluorometer (PSI, Brno), and the fluorescence relaxation traces were analyzed as described earlier [35]. The sample concentration was 10 μg Chl mL^{-1}.

2.5. Thermoluminescence measurements

Thermoluminescence curves were measured with a home built apparatus as described earlier [36]. Samples contained 50 μg Chl, and the measurements were performed in the −40 to +80 °C range with a heating rate of 20 °C min^{-1}.

2.6. Photoinhibition measurements

High-light illumination experiments were performed in open, square glass containers in which the cell suspension formed a 14 mm high layer, with continuous stirring at 25 °C. An array of 50 W halogen lamps with adjustable light intensities provided the homogenous white light illumination of 200 μmol photons m^{-2} s^{-1}. PSII activity was assayed by the initial amplitude of the flash-induced chlorophyll fluorescence signal.

2.7. Protein extraction, SDS-PAGE and immunoblotting

Synechocystis 6803 cells were harvested by centrifugation (10,000*g* for 10 minutes at 4 °C), washed twice with thylakoids buffer (25 mM HEPES/NaOH pH 7, 5 mM $MgCl_2$, 15 mM $CaCl_2$, 10% (v/v) glycerol, 0.5% (v/v) DMSO) and stored at −80 °C until use. Cells were broken in 2 mL screw tubes filled half-way with glass beads. Complete EDTA-free protease inhibitor (Roche, Mannheim, Germany) was added to the suspension. Cells were broken with a BeatBeater (BioSpec. Products) using 8 cycles (30 s each) at maximum speed and cooled on ice for two minutes between each breaking cycle. Glass beads and unbroken cells were separated by centrifuging samples at 600*g* for 5 min at 4 °C. Proteins were determined according to Lowry (1950) modified by Peterson (1977) or with the RC-DC™ protein assay kit (Bio-Rad, Hercules, CA, USA). SDS-page was performed as in Laemmli [37], using 6 M urea in the resolving gel. The same amount of protein (20 μg) was loaded in each lane. Before loading, the samples were warmed at 65 °C for 15 minutes and centrifuged at full speed for 10 minutes in an Eppendorf centrifuge to remove insoluble material. For immunoblotting, proteins were electro-transferred onto PVDF membranes and incubated with primary antibodies. Antisera recognizing the Photosystem II proteins D1, CP43' (IsiA) and allophycocyanin alfa and beta (APC) were received from AgriSera, Sweden, whereas ones recognizing ScpC/D and ScpE were produced in collaboration with AgriSera. The PsbH antibody was a gift from Dr. Komenda, Czech Republic. As secondary antibody, a peroxidase-conjugated goat anti-rabbit IgG was used. Chemoluminescence signals were detected using advance ECL™ western blotting detection system (GE Healthcare, Little Chalfont, Buckinghamshire, England) and quantified using Image J (http://rsbweb.nih.gov/ij/).

2.8. Gabaculine treatment

Cells in exponential phase were diluted to the same OD_{730} and left to grow for 17 h at a light intensity of 2–4 μmol photons m^{-2} s^{-1} in presence of 5 μM gabaculine, an inhibitor of chlorophyll biosynthesis [15]. Cells were then harvested and broken as described in the previous paragraph.

3. Results

3.1. Immunodetection of Photosystem II proteins, allophycocyanin and IsiA

The PSI-less/ScpABCDE^{-} mutant contains approximately three times less chlorophyll per OD_{730} than its PSI-less control [15]. This decrease in chlorophyll content could be due either to a lower content in PSII, or to the absence of other chlorophyll-binding proteins such as SCPs, IsiA [38] or still unknown proteins. Immunostaining of various PSII proteins was therefore used to quantify the amount of these proteins in the two mutant strains. As shown in Fig. 1, the amount of the Photosystem II reaction center protein D1 and PsbH are higher in the PSI-less mutant (Fig. 1, first lane), than in the PSI-less/ScpABCDE^{-} mutant (lane 3) when the same amount of proteins is loaded. PsbH has been shown to be a close neighbor of the SCPs within Photosystem II [31] and also seems to participate in the binding of chlorophyll [39] and the initiation of the PSII repair cycle [40]. As expected, the peptide-directed antibody recognizing an N-terminal sequence of ScpC and ScpD [29] and the one directed against the N-terminus of ScpE did not immunostain any protein in the ScpABCDE-less mutant. While the amount of Photosystem II proteins decreased in the SCP-deficient mutant, the amount of allophycocyanine, a component of the phycobilisome, was constant in both mutants (Fig. 1, lane 5). The expression of IsiA, the iron-stress induced protein, was only minor in both mutants and did not vary between them. As a positive control for the IsiA-recognizing antibody proteins extracted from iron-stressed wild type cells were run in parallel.

3.2. Low temperature fluorescence spectroscopy

Fluorescence spectrometry is a valid method to detect relative differences of pigment-binding proteins, including phycobilisomes. Low temperature (77 K) fluorescence emission spectra offer good resolution of the fluorescence produced by PSII components. Additionally, selection of a special emission wavelength allows relative quantification of the antenna/photosystem ratio. Low-light grown cells with the same chlorophyll concentration were frozen without glycerol to avoid functional uncoupling of the phycobilisomes from thylakoid components and the obtained spectra were normalized to their maximum values. Upon excitation at 435 nm, which excites mainly chlorophyll *a*, two peaks with maximum at 685 and

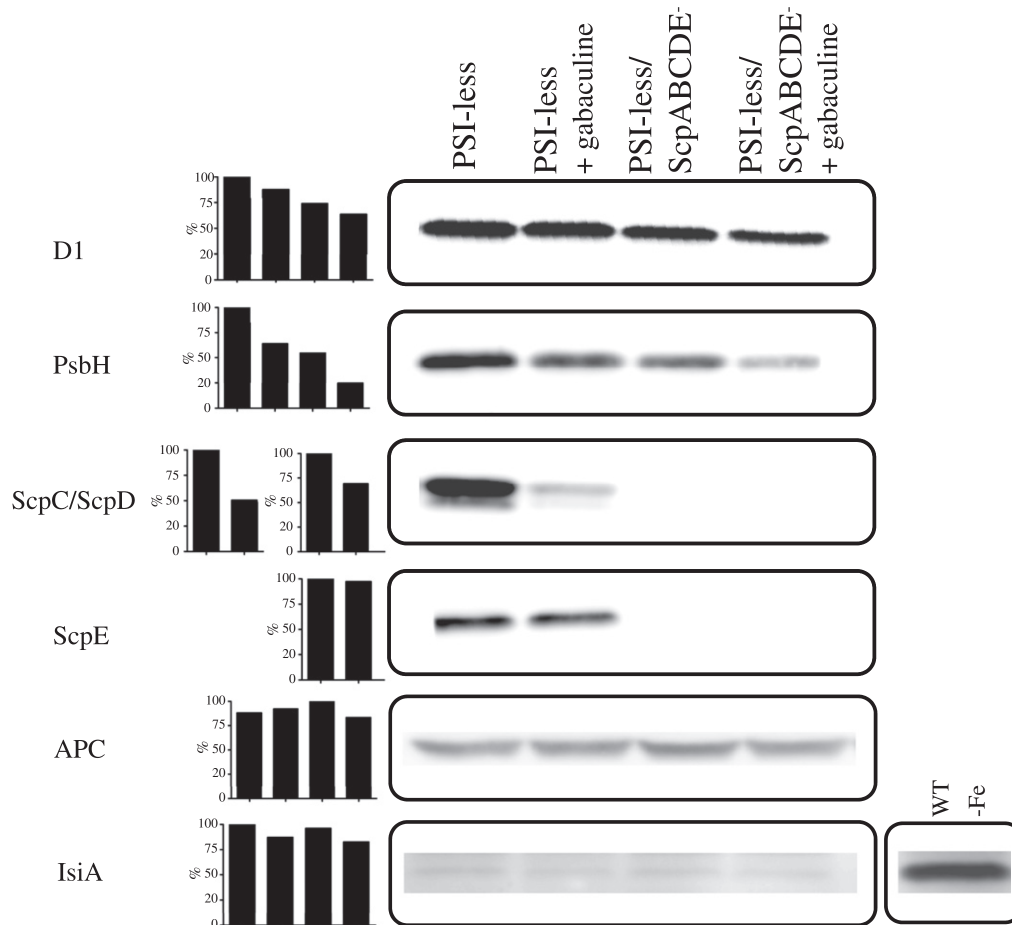

Fig. 1. Immunoblot against total cell extract of the PSI-less and the PSI-less/ScpABCDE^{-} mutants in the presence and absence of gabaculine (5 μM). Antibodies against the Photosystem II proteins D1 and PsbH as well as the ScpC and D, ScpE, allophycocyanin (APC) and IsiA were used. The immunoblots were quantified using Image J (http://rsbweb.nih.gov/ij/).

695 nm were detected. The peak at 685 nm reflects all chlorophyll associated with PSII (including chlorophyll from CP43, D2, D1 and CP47), except for one low energy chlorophyll that appears to be associated with His114 of CP47. This low-energy chlorophyll is the main contributor to the 695 nm emission maximum [41–44]. As seen in Fig. 2A, the 685 nm fluorescence was higher in the *scp*-deletion mutant (dotted line), most likely due to phycobilisomes that are disconnected from the membrane [45]. When cells were excited at 580 nm, light is absorbed mainly by phycobilisomes (Fig. 2B). Emission bands related to phycocyanin (PC, 650 nm), allophycocyanin (APC, 660 nm), the core-linker (Lcm, 685 nm) and PSII (685 and 695 nm) were observed. In the normalized spectra, the 695 nm peak was practically absent in the SCP-depleted mutant compared to the PSI-less control strain, while fluorescence from the 685 nm peak was increased. In iron-stressed cells, chlorophyll bound to IsiA also contributes to the fluorescence peak at 685 nm [46,47]. The accumulation of IsiA can be monitored by a blue shift of the red chlorophyll *a* absorption peak [48], however, neither the PSI-less/ScpABCDE$^-$ mutant nor the PSI-less control displayed this shift, which is consistent with the data obtained by immunoblotting (Fig. 1). The 77 K fluorescence measurements therefore suggest that in the absence of SCPs there are either more PBS per PSII, or the energy transfer from the PBS to PSII is disturbed. The immunoblot results (Fig. 1) showed less Photosystem II proteins, but the same quantity of APC, thus the higher amount of PBS per PSII does not seem to be connected to the photosystem.

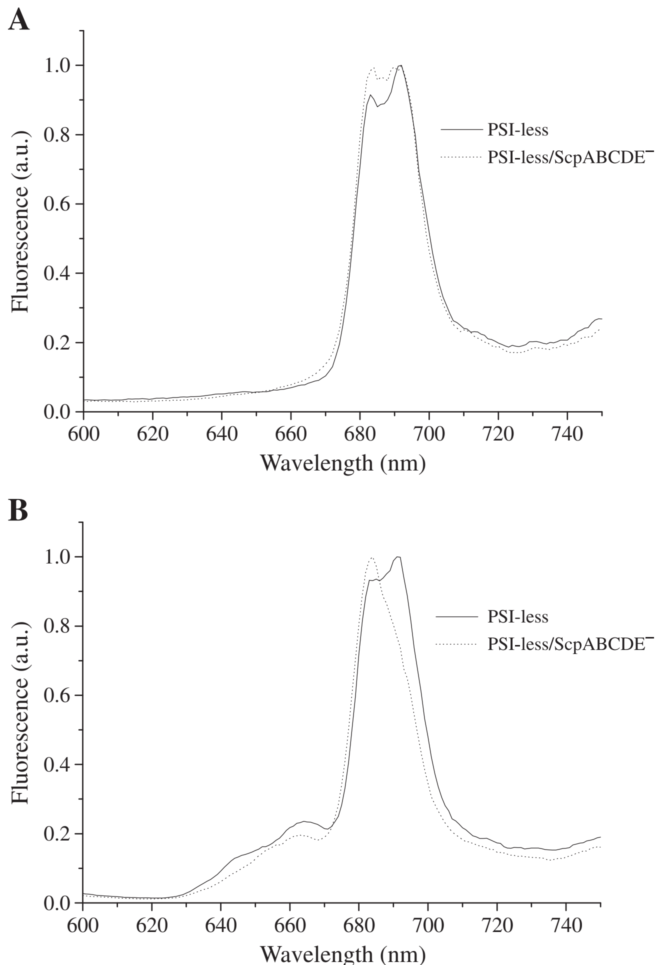

Fig. 2. 77 K fluorescence spectroscopy of the PSI-less mutant (black line) and the PSI-less/ScpABCDE$^-$ mutant (dotted line) from 600 to 750 nm. A. Excitation of chlorophyll at 435 nm. B. Excitation of phycobilisomes at 580 nm.

3.3. Fluorescence induction

To support the previous finding and to investigate whether the PSII antenna size varies in the *scp*-deletion mutant, its fluorescence induction curves were compared with those of the PSI-less control strain. Blue-green light (400–550 nm) is mainly absorbed by chlorophyll, while orange light (max. 600 nm) is absorbed mostly by phycobilins. Under increasing light intensity electrons from PSII reduce the PQ-pool, the photosystem "closes" and the light energy has to be quenched via the release of heat or fluorescence. The amount of fluorescence therefore is directly proportional to the amount of "closed" PSII reaction centers. The inhibitor 3-(3,4-dichloro-phenyl)-1,1-dimethylurea (DCMU) blocks electron transfer between Q_A and Q_B, differences in the antenna size therefore are visualized by the amount of light needed to reach maximum fluorescence. Independent of the wavelength used (Fig. 3A blue light, Fig. 3B orange light) no differences in variable fluorescence kinetics were observed between the two mutant strains containing or depleted of SCPs. Thus, the PSII antenna properties do not seem to be significantly altered in the PSI-

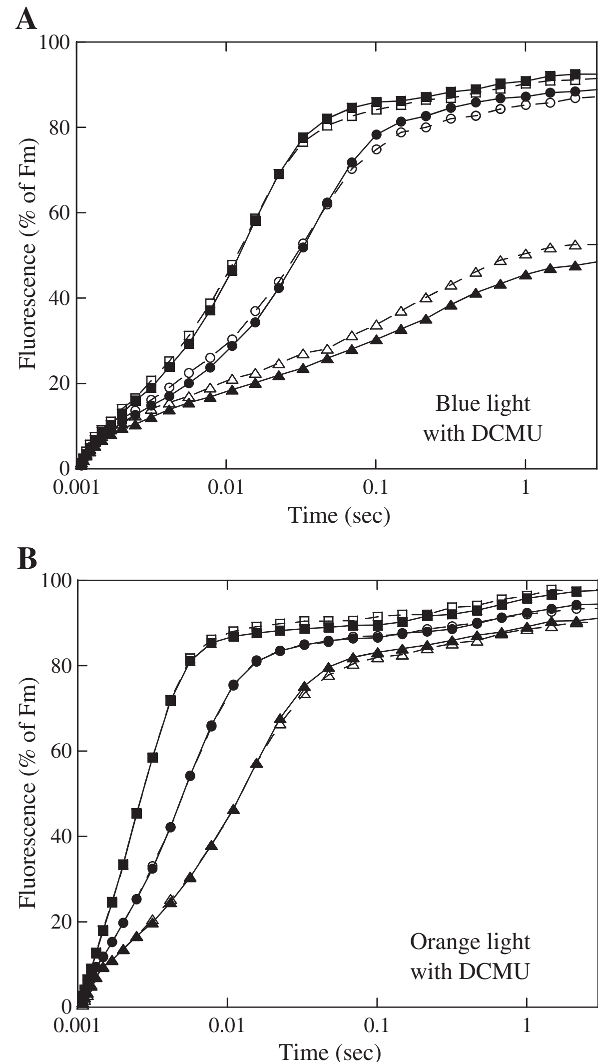

Fig. 3. Fluorescence induction measurements at various light intensities comparing the PSI-less control (open symbols) with the PSI-less/ScpABCDE$^-$ mutant (filled symbols) in the presence of DCMU. A. Excitation with blue light (400 to 550 nm); square: 224 μmol photons m^{-2} s^{-1}; circle: 87 μmol photons m^{-2} s^{-1}, triangle: 8.5 μmol photons m^{-2} s^{-1}. B. Excitation with orange light (600 nm); square: 140 μmol photons m^{-2} s^{-1}, circle: 95 μmol photons m^{-2} s^{-1}, triangle 26 μmol photons m^{-2} s^{-1}.

less/ScpABCDE mutant, neither when chlorophyll nor phycobilins are excited. Even though more phycobilisomes per PSII were found in this mutant (Figs. 1 and 2), they do not correspond to a higher number of PBS connected to PSII.

3.4. OCP related non-photochemical quenching

SCPs, similar to plant Lil proteins, are thought to be involved in NPQ [8,32]. Contrary to plants, evidence is still missing on their role in NPQ in cyanobacteria. In *Synechocystis* 6803, the main player in NPQ is the photoactive orange carotenoid protein (OCP) (reviewed by Kirilovsky [49]). Absorption of blue-green light induces conformational changes in the carotenoid and the protein converting the stable orange dark OCP form (OCP^o) into a metastable red active form (OCP^r) [50]. Under high irradiance, OCP^r induces the decrease of phycobilisome fluorescence and the energy arriving at the reaction centers by increasing energy thermal dissipation [51]. To study whether the blue-light-induced fluorescence quenching related to OCP also depends on the presence of the SCPs, fluorescence emission derived from chlorophyll and phycobilins was monitored by a pulse-amplitude modulated (PAM) fluorometry [52]. Dark-adapted cells with the same chlorophyll concentration were illuminated first with dim blue light for 50 s, then with strong blue-green light for 100 s and then allowed to recover in non-actinic measuring light (Fig. 4A, B). The initial fluorescence (F_0) in dark adapted cells was lower in the PSI-less control (Fig. 4A) than in the PSI-less/ScpABCDE- mutant (Fig. 4B), pointing to functionally disconnected PBS. Upon weak blue light illumination the fluorescence increased to a new stable basal fluorescence, Fs. The amount of PBS quenched fluorescence was monitored during exposure to strong blue-green light. As visible in Fig. 4B, quenching was considerably higher in the absence of SCPs, indicating either a higher content of OCP per PBS or an overall higher content (per chlorophyll) of both PBS and OCP in the *scp*-mutant. In light of the previous results, the second hypothesis is more likely. However, while the phycobilisomes can bind the activated OCP (their fluorescence is quenched) [52,53], they are disconnected from PSII. Our data show that despite their homology to plant Lil proteins the SCPs are not involved in cyanobacterial NPQ.

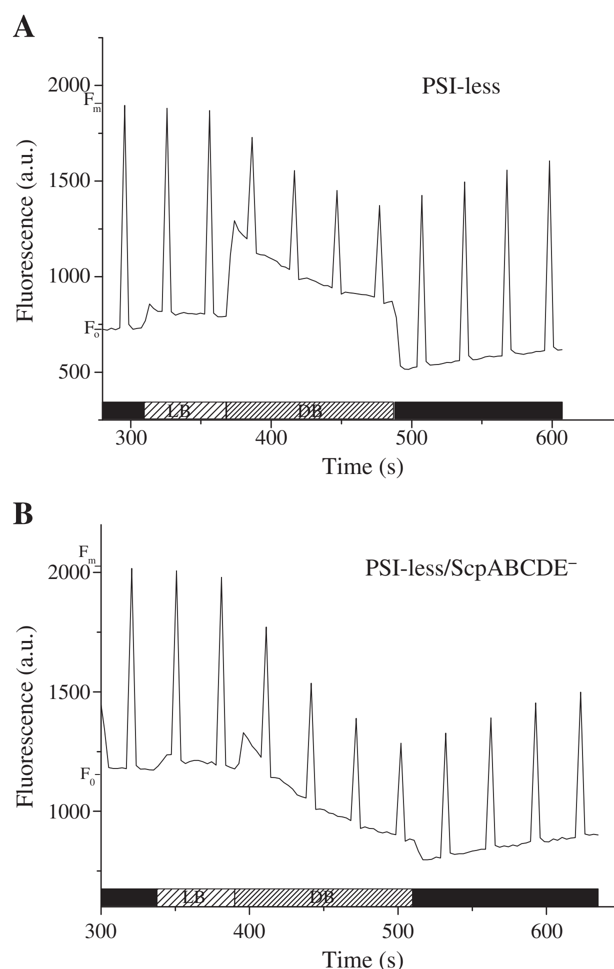

Fig. 4. Non-photochemical quenching of the PSI-less strain (A) and the PSI-less/ScpABCDE$^-$ (B) mutant using PAM chlorophyll fluorescence. Dark-adapted cells with the same chlorophyll concentration were illuminated first with dim blue light for 50 s (LB), then with strong blue-green light for 100 s (DB) and then allowed to recover in non-actinic measuring light (dark bar).

3.5. Thermoluminescence measurements

Previously it was shown that even when SCPs can attach to PSII [29,30] the absence of SCPs do not alter the light-harvesting capacity of PSII [16,21]. Therefore the lack of SCPs might lead to structural rearrangements within the Photosystem II complex. Thermoluminescence (TL) has proven to be a very sensitive and reliable biophysical method for investigating functional PSII donor and acceptor side components [54,55]. TL signals result from the thermally activated recombination of trapped electrons and stabilized positive "holes" on the reduced quinone acceptors (Q_A or Q_B) and on the S_2 or S_3 oxidation state of the water-splitting complex, respectively. PSI-less *Synechocystis* cells were compared to PSI-less/ScpABCDE$^-$ cells using TL in the absence (Fig. 5A) or presence (Fig. 5B) of DCMU. In the absence of DCMU, illumination of dark adapted material with a single flash generates an $S_2Q_B^-$ charge pair, which induces the so-called B band of TL light emission (Fig. 5A). Compared to the PSI-less control, the PSI-less/ScpABCDE$^-$ strain displayed a significant difference (of about 7 °C) in the position of the B band (Table 1); while the Q band (S_2Q_A-recombination), visible in the presence of DCMU (Fig. 5B, Table 1) was similar to the control. An additional band was seen in the spectra at around 52–55 °C; in the PSI-less control it had a low amplitude in the absence of DCMU (Fig. 5A), which increased in the presence of DCMU (Fig. 5B). Therefore, this band can most easily be assigned to the so-called C band, which arises from the $Tyr^{-}D^{+}Q_A^-$ recombination. At a similar position, a band could also be observed in the SCP depleted mutant. However, its amplitude before normalization was the same in the presence and absence of DCMU (not shown). This may indicate that this band at 52–55 °C in the SCP-deletion mutant arose from a population of PSII centers, in which the Q_A to Q_B electron transfer was permanently blocked. It seems therefore that the Q_B site is modified in the absence of SCPs.

3.6. Flash-induced chlorophyll fluorescence measurements

Flash-induced chlorophyll fluorescence measurements can be used to monitor forward electron transfer, as well as charge recombination processes of the reduced Q_A and Q_B acceptors. Illumination of *Synechocystis* cells with a single turnover saturating flash induces the reduction of Q_A, leading to increased fluorescence yield. Subsequent reoxidation of Q_A^- in the dark results in the relaxation of fluorescence yield, exhibiting three main decay phases [56–58]. The fast phase of fluorescence decay arises from the reoxidation of Q_A^- by PQ molecules bound to the Q_B site, either in the oxidized or in the semi-reduced state, before the flash. The middle phase originates from Q_A^- reoxidation by PQ molecules in centers, where the Q_B site was empty at the time of the flash. Finally, the slow phase arises from the back reaction of the S_2 state of the water-oxidizing complex with Q_A^-, which is populated via the equilibrium between $Q_A^-Q_B$ and $Q_AQ_B^-$ [59]. As seen in Fig. 6, the PSI-less strain and the PSI-less/ScpABCDE$^-$ cells show identical decay times for the $S_2Q_A^-$

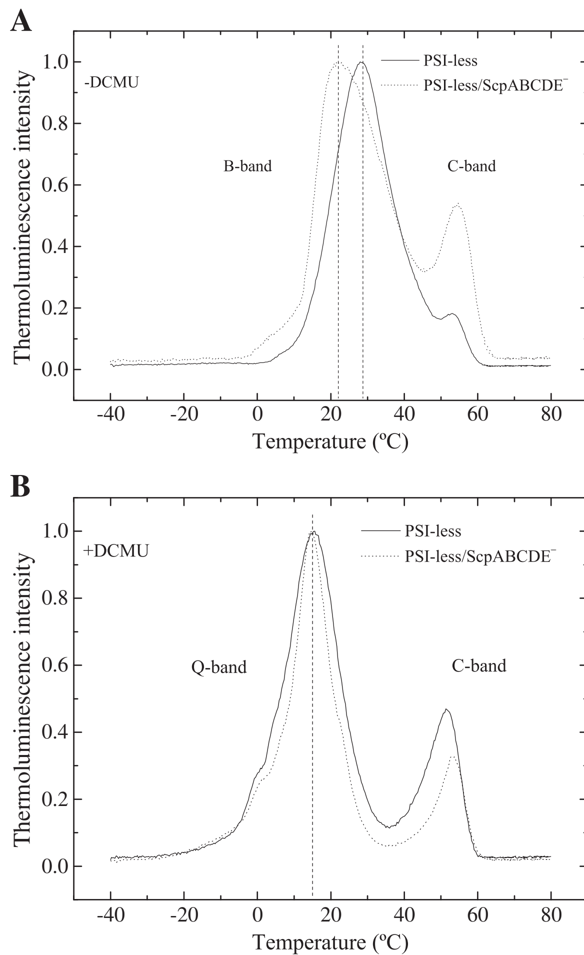

Fig. 5. Thermoluminescence emission from PSI-less strain (black line) and the PSI-less/ScpABCDE^{-} mutant (dotted line) in the absence (A) and presence (B) of DCMU. Temperature differences are given in Table 1.

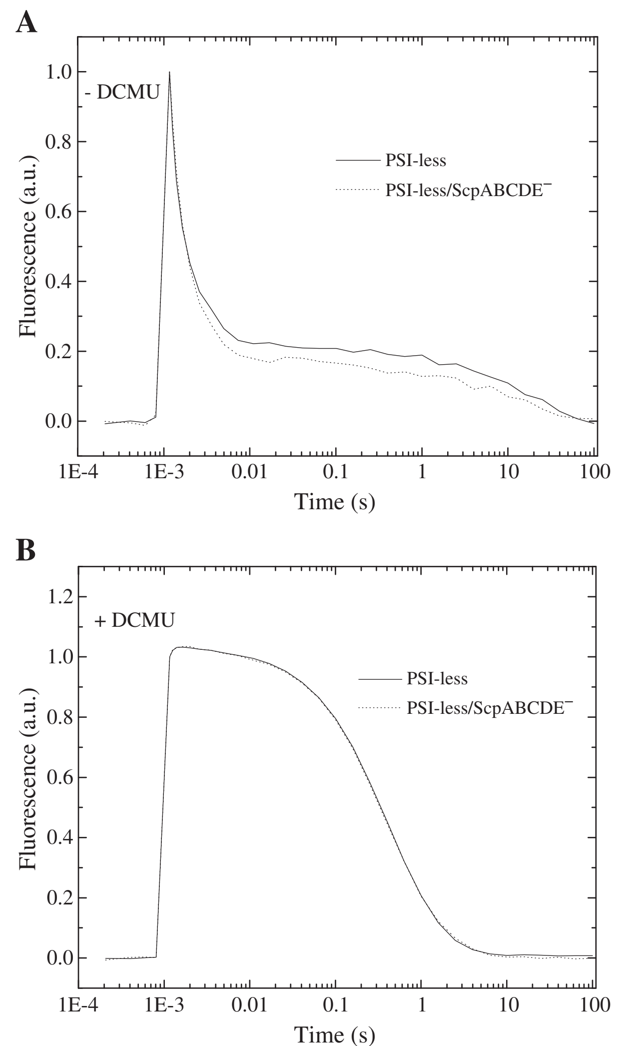

Fig. 6. Flash-induced chlorophyll fluorescence measured on the PSI-less (black line) and the PSI-less/ScpABCDE^{-} strain (dotted line) in the absence (A) and presence (B) of DCMU. Differences in kinetics are given in Table 2.

recombination (0.53 s in the presence of DCMU, Fig. 6B, Table 2). However, in the absence of DCMU (Fig. 6A) a faster decay for $S_2Q_B^-$ was observed in the PSI-less/ScpABCDE^{-}mutant (6.3 s) than in the PSI-less control (8.4 s) in the slow phase (Table 2). The $S_2Q_B^-$ recombination therefore is accelerated in the SCP-deficient mutant, but the $S_2Q_A^-$ recombination is not. The differential acceleration of the $S_2Q_B^-$ recombination relative to that of $S_2Q_A^-$ is in agreement with the TL data and shows a decreased redox gap between Q_A and Q_B due to the shift of the redox potential of Q_B/Q_B^- to more negative values; which indicates a modification of the Q_B binding site. This idea is in agreement with the fast phase of the fluorescence decay being slower in the PSI-less/ScpABCDE^{-} mutant (360 μs) than in the PSI-less strain (270 μs), since the decreased redox gap between Q_A and Q_B represents a smaller driving force for the forward electron transfer between Q_A^- and Q_B.

3.7. Photoinhibition

After observing that absence of SCPs led to changes in the Q_B site of Photosystem II, we were interested in investigating their functional implications. It was reported earlier, that the lifetime of chlorophyll is dependent on the presence of SCPs [25,27]. We now wanted to investigate if the SCPs also are important for the recycling of chlorophyll during the repair cycle of PSII, which occurs in response to photoinhibitory damage. Cultures of PSI-less and PSI-less/ScpABCDE^{-} cells were therefore treated with gabaculine, an inhibitor of the chlorophyll synthesis. Before this treatment, each culture was divided into two fractions, to one of them gabaculine was added, the other fraction was

Table 1
Temperature differences obtained by thermoluminscence measurements on the PSI-less control strain and the PSI-less/ScpABCDE^{-} mutant in the presence and absence of DCMU.

Strain	DCMU		+ DCMU	
	B band ($S_2Q_B^-$) *Tm* (°C)	C band ($Tyr_DQ_A^-$) *Tm* (°C)	Q band ($S_2Q_A^-$) *Tm* (°C)	C band ($Tyr_DQ_A^-$) *Tm* (°C)
PSI-less	29 ± 0.5	53 ± 0.5	15 ± 0.8	51 ± 0.5
PSI-less/ScpABCDE^{-}	22 ± 0.6	55 ± 0.5	15 ± 0.7	53 ± 0.6

Table 2
Differences in kinetics obtained by flash-induced chlorophyll fluorescence measurements on the PSI-less and the PSI-less/ScpABCDE$^-$ strain in the presence and absence of DCMU.

Strain	Fast phase	Middle phase	Slow phase
	T 1/Amp (ms/%)	*T* 2/Amp (ms/%)	*T* 3/Amp (s/%)
No addition			
PSI-less	0.27 ± 0.03/61 ± 3	2.2 ± 0.2/24 ± 3.4	8.4 ± 0.7/15 ± 0.3
PSI-less/ ScpABCDE−	0.36 ± 0.04/62 ± 4	2.2 ± 0.3/26 ± 5	6.3 ± 0.7/12 ± 0.3
+ DCMU			
PSI-less	1/0	10/0	0.52 ± 0.2/100 ± 0.8
PSI-less/ ScpABCDE−	1/0	10/0	0.53 ± 0.3/100 ± 0.8

kept as control. Both fractions were grown for 17 h and then resuspended to the same chlorophyll concentration. Photoinhibitory treatment was performed at 200 µmol photons m^{-2} s^{-1} for 90 min, recovery was allowed at 4 µmol photons m^{-2} s^{-1}for another 90 min. As shown in Fig. 7A, in the PSI-less strain gabaculine addition induced a significant acceleration of photoinhibitory loss of PSII activity (empty circles), which was quantified by the initial amplitude of the flash-induced chlorophyll fluorescence signal. In addition, the activity loss of PSII could only be partially restored during a recovery period in weak light when gabaculine was present (empty circles), whereas the recovery was almost complete in the absence of gabaculine (empty squares). The enhanced loss of PSII in the presence of gabaculine is related to the partial impairment of PSII repair, since elimination of protein synthesis by the inhibitor lincomycin leads to identical rate and extent of PSII activity loss in the presence and absence of gabaculine (filled squares and circles).

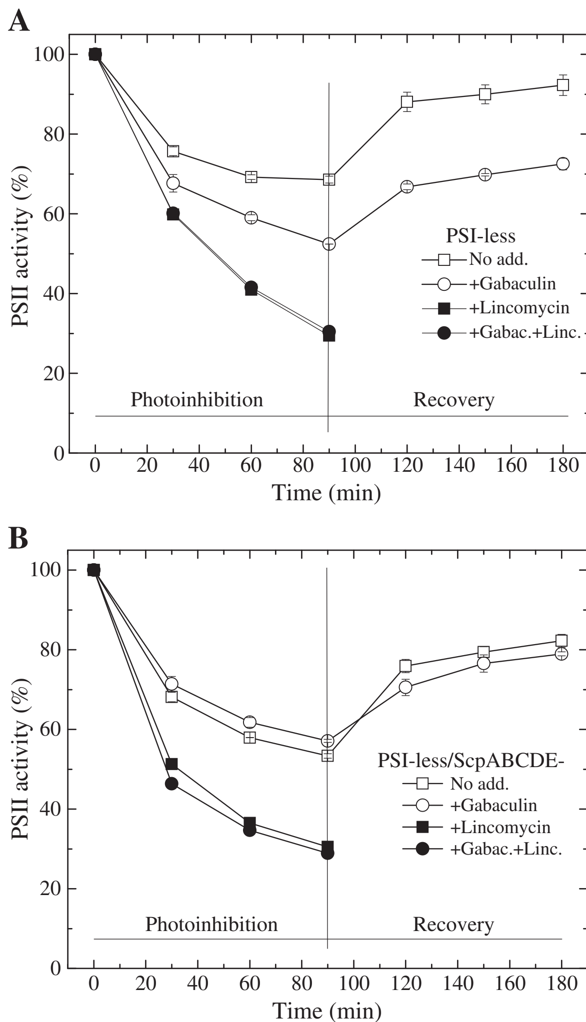

Fig. 7. Protein- and chlorophyll-synthesis dependent photodamage and repair of PSII in the PSI-less (A) and the PSI-less/ScpABCDE$^-$ strain (B). PSII activity was quantified by the initial amplitude of flash-induced chlorophyll fluorescence signal. Photoinhibitory treatment was performed in the presence (circles) or absence (squares) of gabaculine (5 µM), as well as in the presence (closed symbols) and absence (empty symbols) of 300 µg/mL lincomycin. Chlorophyll concentration was adjusted after gabaculine incubation (for 17 h) prior to measurement. High light (200 µmol photons m^{-2} s^{-1}) and recovery light (4 µmol photons m^{-2} s^{-1}) were applied for 90 min.

In the PSI-less/ScpABCDE$^-$ mutant, gabaculine addition had no effect on PSII damage, neither when measured in the presence (Fig. 7B, filled squares and circles) nor in the absence of lincomycin (Fig. 7B, empty squares and circles). In addition, the rate of PSII activity loss was the same in the PSI-less/ScpABCDE$^-$ mutant in the absence of gabaculine (Fig 7B, empty squares), as in the PSI-less strain in the presence of gabaculine (Fig.7A, empty circles). This result indicates that chlorophyll synthesis is required for efficient PSII repair, and also that chlorophyll synthesis is partly impaired in the absence of the SCPs. A reduction of protein amount due to gabaculine treatment was also observed by immunoblotting (Fig. 1). In the PSI-less, as well as in the PSI-less/ScpABCDE$^-$ mutant, the amount of the Photosystem II proteins D1 and PsbH was reduced (by 30% and almost 50%, respectively) in the presence of gabaculine compared with untreated cells. Chlorophyll deficiency also reduced the amount of IsiA (Fig. 1, lanes 2 and 4). One should note that in the PSI-less strain, ScpC and D are reduced to a much larger extent (by 50% and 30%, respectively), while the amount of ScpE does not change at all (Fig. 3, lanes 1 and 2). Gabaculine had no effect on the amount of allophycocyanin indicating its stability over the time span of the treatment (Fig. 1).

4. Discussion

4.1. SCPs guide the assembly of PSII

The small CAB-like proteins belong to the light-harvesting-like proteins and are only induced by stress in *Synechocystis* 6803 [57]. Deletion of several or all *scp* genes in a wild type background had little or no effect on the cell phenotype grown under standard laboratory conditions [15,21]. Similar results were observed, when SCPs expression was suppressed in mutants lacking PSII [27]. Contrary in mutants lacking PSI, the absence of SCPs impaired cell growth and reduced the amount and half-life of chlorophyll molecules [15,25,26]. In *Synechocystis* 6803 approximately 80% of the chlorophyll is associated with Photosystem I [33]. A mutant lacking both Photosystem I and Photosystem II still contains 4% chlorophyll per cell compared with wild type [60]. Deletion of SCPs further reduced the amount of chlorophyll per cell [15]. Immunological and microscopy analyses identified ScpB, C and D to be associated with Photosystem II under stress conditions [29,31]. This, together with the ability of SCPs to bind chlorophyll in vitro [19] reinforces the proposed role of SCPs as stabilizers of chlorophyll freed during assembly or repair of Photosystem II. These data justify the use of a PSI-less background mutant, in which the SCPs are constitutively expressed [21]. Since deletion of single SCPs genes has little effect on the overall cell phenotype [16] and to exclude overlapping functions of these highly homologous proteins [21], we compared the PSI-less control strain [61] with a PSI-less mutant, in which all 5 SCPs were deleted [15].

It was observed previously that deletion of all SCPs in the PSI-less background reduces the amount of both chlorophyll and carotenoids drastically [15,16]. Using immunoblotting we were able to show that the reduction of chlorophyll is accompanied by a reduced amount of PSII in the cells depleted of SCPs. Interestingly, while the PSII amount is reduced (and PSI is absent in these mutants), the amount of phycobilisomes stays constant in the cell (Figs. 1, 2 and 3). However,

this larger proportion of phycobilisomes per PSII does not result in a higher energy transfer between both complexes, instead they seem to be disconnected from PSII. These disconnected phycobilisomes can still bind the activated OCP and their fluorescence is quenched under strong irradiance similarly to PSII attached phycobilisomes. It has previously been shown that SCPs are not involved in light-harvesting [16,21] and therefore do not connect PBS to the PSII-core. The higher amount of PBS per PSII in the absence of SCPs could therefore be explained by a miss-regulation of the tetrapyrrole biosynthetic pathway and/or PSII assembly/repair machinery [16].

Depletion of SCPs did not only induce a decrease of PSII per cell, but also structural changes within the Photosystem. ScpD and probably also ScpC are known to bind to CP47 [29] and/or to PsbH, at the site of Photosystem II, where PQ molecules enter [31]. By thermoluminscence and flash-induced chlorophyll fluorescence measurements we were able to monitor changes at the Q_B site in the absence of SCPs. In a population of PSII, the transfer of electrons between Q_A and Q_B was permanently blocked. Previously published studies have shown SCPs to be associated with PSII complexes during assembly and under stress conditions (29, 31). In the light of our results SCPs appear to function as molecular chaperones assisting in the assembly and repair of PSII.

4.2. SCPs are not involved in non-photochemical quenching

Cyanobacterial NPQ happens at the site of the phycobilisomes and involves the orange carotenoid protein [51]. Due to their homology to plant Lil proteins, SCPs also have been proposed to be involved in NPQ [8,32], however, evidence for this role is still missing. Our data show that in the PSI-less/ScpABCDE$^-$ mutant there is a higher amount of PBS-OCP per PSII compared with the control, and in the absence of SCPs, the cells performed higher NPQ (Fig. 4). While the higher NPQ is a result of the larger amount of PBS-OCP, a participation of SCPs in this process is very unlikely. Furthermore, SCPs depletion did not induce IsiA accumulation (Fig. 1). These results are in agreement with previously published results showing that IsiA also is not involved in OCP-related NPQ [45].

4.3. SCPs stabilize chlorophyll

The half-life of chlorophyll in SCP-deletion mutants has been shown to be shorter compared with control strains [25]. SCPs seem to preferentially stabilize PSII-associated chlorophylls, but not the PSII complex itself [27]. These findings raise the question, whether SCPs are directly involved in the stabilization of pigment—by binding free chlorophyll originating from damaged PSII (*in vitro* these proteins are able to bind chlorophyll [19]), and/or if they are indirectly involved in the process—by regulating the tetrapyrrole metabolism [15]. To investigate the role of SCPs in the repair cycle of PSII, we exposed the cells to gabaculine and examined the recovery of the photosynthetic activity after photobleaching. Gabaculine inhibits the synthesis of 5-aminolevulinic acid and thus the synthesis of chlorophyll [62,63]. Hence, cells grown in the presence of gabaculine can only repair or assemble new PSII with chlorophyll molecules recycled from damaged PSII. As expected, the presence of gabaculine enhanced photoinhibition in the PSI-less control. However, after photoinhibitory treatment a low recovery rate was observed, indicating chlorophyll recycling into newly synthesized PSII. When gabaculine was used in combination with lincomycin, an inhibitor of protein synthesis, significant differences were observed in the PSI-less mutant, confirming that chlorophyll synthesis is needed for the assembly of new PSII. In the absence of SCPs, however, severe photoinhibition was already observed in untreated cells (similarly to the PSI-less strain in the presence of gabaculine), addition of gabaculine had no additional effect on photoinhibition and recovery. SCPs, therefore, seem to be involved in the repair cycle of PSII by regulating the synthesis of new chlorophyll. Interestingly, not only the chlorophyll synthesis seems to be affected by the lack of SCPs, but the amount of chlorophyll available in the cell also seems to regulate SCP expression, at least that of ScpC/D. In the PSI-less control strain, the amount of ScpC/D was reduced in gabaculine treated cells, but the amount of ScpE stayed the same, independent of the treatment. Previously we showed that deletion of the *chl*L gene (essential for chlorophyll synthesis in the dark) reduced the expression of SCPs in mutants lacking PSI, when grown in light activated heterotrophic growth (LAHG) conditions [21]. Based on the observations that i) SCPs attach to PSII *in vivo*, ii) are able to bind chlorophyll *in vitro* and iii) their absence alters the chlorophyll metabolism, we propose that SCPs are involved in the coordination of chlorophyll biosynthesis during PSII assembly/turnover, a role that is important during oxidative stress.

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