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Deletion of the gene family of small chlorophyll-binding proteins (ScpABCDE) offsets C/N homeostasis in *Synechocystis* PCC 6803☆

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ABSTRACT

In the family of chlorophyll binding proteins, single helix small CAB-like proteins (SCPs) are found in all organisms performing oxygenic photosynthesis. Here, we investigated the function of these stress-inducible proteins in the cyanobacterium *Synechocystis* sp. PCC 6803. We compared physiological, proteome and transcriptome traits of a Photosystem I (PSI) deletion strain, which constitutively induces SCPs, and a PSI-less/ScpABCDE^{-} without SCPs. The SCP mutant cells were larger in size, showed irregular thylakoid structure and differed in cell-surface morphology. Deletion of *scp* genes strongly affected the carbon (C) and nitrogen (N) balance, resulting in accumulation of carbohydrates and a decrease in N-rich compounds (proteins and chlorophyll). Data from transcriptomic and metabolomic experiments revealed a role of SCPs in the control of chlorophyll biosynthesis. Additionally, SCPs diminished formation of reactive oxygen species, thereby preventing damage within Photosystem II. We conclude that the lack of SCP-function to remove free chlorophyll under stress conditions has a large impact on the metabolism of the entire cell.

1. Introduction

During photosynthesis sunlight is converted into chemical energy. This process occurs within the thylakoid membrane, where pigments bound to specialized antennae absorb and transfer light energy to a reaction center (RC). Cyanobacteria are the earliest known oxygenic photosynthetic organisms. They are now the focus for large-scale photosynthetic production of renewable biofuels and other products [1]. Efficient production of biomass by photosynthesis requires strong enough light, however, this requirement goes hand in hand with simultaneous damage to key components of the photosynthetic machinery, especially in Photosystem II (PSII) [2]. Adaptation to stress situations requires readjustment of homeostasis on both molecular and cellular levels. Plants, algae and cyanobacteria have developed several mechanisms to protect themselves against light-induced damage. In cyanobacteria, the orange carotenoid protein in its active red form promotes the non-photochemical quenching (NPQ) of energy absorbed by PSII and the phycobilisomes—the typical light harvesting antennae present in cyanobacteria (reviewed in [3]).

The close connection between light-harvesting and light-damage prevention is illustrated by the homology between the higher plant chlorophyll *a*/*b* binding (CAB) antenna proteins and the light-harvesting like (Lil) proteins, which are involved in light protection of plants and cyanobacteria [4]. Similar to the higher plant antenna proteins, Lil proteins contain a CAB domain. However, in contrast to CAB antenna proteins, Lil proteins are up-regulated under high light, and are participating in NPQ (as shown for PsbS [5]), otherwise Lil proteins are affecting pigment stability and/or are acting as pigment carriers [6–9]. Unlike higher plants, cyanobacteria only contain low molecular weight Lil proteins, consisting of one or two membrane-spanning helices. Because of their enhanced expression under high light, these Lil proteins are also called HLIPs (high light induced proteins, after [10]). In *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803), five Lil proteins were identified [11]. They were actually described as small CAB-like proteins (SCPs, and their individual genes named *scpA–E*) [11], since they were induced not only during high light, but also during other stress conditions [86]. ScpA is the C-terminal extension of the ferrochelatase HemH, an enzyme involved in heme biosynthesis [11–

Abbreviations: CAB domain, chlorophyll *a*/*b* binding domain; FC, fold change; FDR, false discovery rate; FTIR, Fourier Transform Infrared Spectroscopy; HLIP, high light induced protein; LIL, light-harvesting like protein; NPQ, non-photochemical quenching; ROS, reactive oxygen species; SCP, small CAB-like protein.

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14]; while ScpB–E are proteins of around 6 kDa with a single transmembrane helix and are functionally and physically associated with PSII [15–18].

Similar to Lil proteins in plants, SCPs have been proposed to 1) function in light dissipation [19], 2) to act as chlorophyll carriers during assembly/repair of PSII [8,9,20,21], or 3) to regulate chlorophyll biosynthesis [22,23], reviewed in [24]. While inactivation of single *scp* genes did not lead to obvious phenotypes [11], multiple *scp* deletions in *Synechocystis* 6803 altered cell pigmentation [22] and decreased the amount of chlorophyll, carotenoids, and phycobilisomes [19,23] on exposure to stress. A mutant lacking *scpBCDE* was unable to survive in high light and compared to WT showed impaired ability to perform NPQ [19]. SCPs are only stress-induced in wild type, conversely they are constitutively expressed in mutants deficient of Photosystem I (PSI) [11]. The phenotype observed upon deletion of all five *scp* genes was therefore optimally contrasting that of the constitutively induced SCP presence in mutants lacking PSI [22,23]. For clarity, it is stated that in the wild type *scp* genes are not constitutively expressed, hence the PSI-less excellently serves the goal of this study to reveal the role of SCP proteins. The PSI-less/ScpABCDE$^-$ mutant (hereafter SCP mutant) of *Synechocystis* 6803 contained only one third of the chlorophyll amount of a PSI-less control [22,23] and the half-life of its chlorophyll molecules was shortened 5-fold [25]. The pronounced impact of SCPs on the physiology of PSI-less background mutants and their association with PSII suggest that SCPs play a role in stabilization of PSII-associated chlorophyll [8,9,15–17], whereas it has otherwise been observed that they do not significantly alter the lifetime of chlorophyll associated with PSI [25]. In recent years, various articles have resulted from the use of mutants lacking PSI to investigate SCPs' function [9,46]. Here, we note that the constitutive expression of SCPs, and the presence of PSII only in the vicinity of which SCPs are localized, is ideal for our study.

Here, we investigated the functional role of SCPs in carbon/nitrogen (C/N) homeostasis. Using the *Synechocystis* 6803 strain depleted of PSI, with constitutive expression of SCPs [11], called control mutant; and the SCP mutant strain with deletion of all five *scp* genes in PSI-less background. Our results show pleiotropic effect of SCPs deletion in *Synechocystis* 6803 on cell morphology and C/N metabolism supporting the proposed role of SCPs in two converging processes: photosystem assembly and chlorophyll biosynthesis. Electron microscopy analysis revealed alteration of the cellular organization of *Synechocystis* 6803 in the SCP mutant, where cells presented a rougher surface morphology compared to the control. Full genome DNA-microarray studies indicated that expression of genes involved in nitrogen assimilation and glutamate metabolism was significantly affected. This observation was also supported by FTIR and metabolomic studies showing that deletion of the *scp* genes had a strong impact on the C/N balance of the cell with accumulation of carbohydrate reserves and decreased abundance of N-rich compounds.

2. Materials and methods

2.1. Growth conditions and cell counting

Synechocystis 6803 PSI-less control [26] and SCP (PSI-less/ScpABCDE$^-$ [23]) mutants were cultivated at 30 °C at a light intensity of 4–5 μmol photons m^{-2} s^{-1} in BG-11 growth medium [27] supplemented with 10 mM glucose. To reduce the risk of secondary mutations that could affect the phenotype, cultures were renewed every three months starting from glycerol stocks. Optical density at 730 nm (OD_{730}) measured with a T90+ spectrophotometer (PG Instruments Ltd., UK) was used to monitor growth. Cell counting was carried out using a Neubauer improved chamber or a Multisizer™ Coulter Counter^R (Beckman Coulter Inc., USA). Cultures were harvested between 5 x 10^8 and 10^9 cell mL^{-1}. Experiments with added α-ketoglutarate, glutamate, and pyruvate were carried out at final concentrations of 10 mM for each compound dissolved in growth medium buffered with 20 mM TES-NaOH at pH 8.2. Pigment extraction and quantification were carried out after four days.

2.2. Pigment determination

Chlorophyll *a* (hereafter chlorophyll) was extracted using 100% methanol. Its concentration was determined from the absorbance at 665 nm, using the extinction coefficient 71.43 mM^{-1} cm^{-1} after [28]. Carotenoid concentration was estimated in a dimethyl-formamide extract after [29]. The amount of phycocyanin was measured, as in [22].

2.3. Protein oxidation assay

The level of protein oxidation was estimated using the OxyBlot™ Protein Oxidation Detection Kit (EMD Millipore, MA, USA) except that samples were solubilized in dim light with 7.5% SDS (final concentration) for 30 min at room temperature.

2.4. Determination of carbohydrate and total protein amount

Cell pellets were resuspended and broken in 0.5 M $HClO_4$ by bead-beating (6 cycles, each 30 s, with 5 min cooling between cycles). Total carbohydrates were measured after [30,31]. Total protein was estimated after acetone precipitation as in [32].

2.5. Optical microscopy

Cells were analyzed with a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy, USA) equipped with a 100× oil immersion lens; pictures were taken with an AxioVision camera. Cell size was estimated from four biological replicates. The diameters of 100 cells per replicate were measured using AxioVision 4.8 software.

2.6. Electron microscopy

For scanning electron microscopy (SEM), cells were fixed in the dark for 1 h at 4 °C using 2.5% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.2) and post-fixed in 1% osmium tetraoxide in a cacodylate buffer for 2 h. Cells were rinsed, snap-frozen in liquid nitrogen and freeze-dried overnight. Specimens were attached to aluminum specimen holders with double-sided carbon tape (Agar Scientific Ltd., UK.) and coated with 10 nm of gold in a modified vacuum coating system equipped with an automated tilting and rotating device (Edvards High Vacuum Ltd., UK). Analysis was carried out using a Cambridge S360ixp SEM (Cambridge Scientific Instruments Ltd., UK.) operated with a LaB6 gun. For transmission electron microscopy (TEM) cells were prepared and analyzed as in [33].

2.7. Metabolomics

Biological replicates of each mutant were collected by harvesting 1 L per replicate; 17 replicates were harvested for the PSI-less control, and 15 for SCP mutant. Cell pellets were washed, frozen in liquid N, freeze-dried, divided into 30 mg aliquots, and stored at −80 °C until metabolite extraction. Metabolite extraction, derivatization, and GC-MS analysis were performed after [34] and [35,36]. Data were normalized by dividing the peak area, with one from Succinic acid (internal standard), and by the number of cells. Data were processed and evaluated using SIMCA software (Umetrics AB, Sweden). Classification and modeling was carried out using orthogonal partial least square discriminant analysis. A list of significant metabolites based on a one-tail *t*-test is given in Supplementary Table 1. An in-house database and the Golm Metabolome Database (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html) were used to identify metabolites.