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Incorporation of the chlorophyll *d*-binding light-harvesting protein from *Acaryochloris marina* and its localization within the photosynthetic apparatus of *Synechocystis* sp. PCC6803

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ABSTRACT

The gene encoding a chlorophyll *d*-binding light-harvesting protein, *pcbA* from *Acaryochloris marina* (now called as accessory Chlorophyll Binding Protein CBPII) marked with a His-tag was transformed into the genome of *Synechocystis* PCC6803. Protein gel electrophoresis and western blotting confirmed that this foreign chlorophyll *d*-binding protein CBPII was expressed and integrated into the thylakoid membrane and bound with chlorophyll *a*, the only type of chlorophyll present in *Synechocystis* PCC 6803. Native electrophoresis suggested that CBPII interacts with photosystem II of *Synechocystis* PCC 6803. Surprisingly, spectral analyses showed that the phycobiliproteins were suppressed in the transformed *Synechocystis* *pcbA*$^{+}$, with a lower ratio of phycobilins to chlorophyll *a*. These results suggest that there are competitive interactions between the external antenna system of phycobiliproteins and the integral antenna system of chlorophyll-bound protein complexes.

1. Introduction

The unusual cyanobacterium *Acaryochloris marina* uses chlorophyll (Chl) *d* as its major photopigment [1,2]. Chl *d* allows *Acaryochloris* the critical advantage of using longer wavelengths of light (690–750 nm) that are not absorbed by organisms containing other Chls [3]. A major challenge is now posed to our understanding: Chl *a* is the predominant pigment in nearly all oxygenic photosynthetic organisms from cyanobacteria to land plants, whereas its function is mostly replaced by Chl *d* in *Acaryochloris*. Introducing the Chl *d*-bound light-harvesting protein into a Chl *a*-containing cyanobacterium will not only permit investigation of the mechanism of chlorophyll replacement, but will also provide a system for exploring the functional and evolutionary development of various light-harvesting strategies in photosynthetic organisms.

Harvesting light energy from the sun is the first step of photosynthesis. The harvested light energy then is funnelled to a trap or photosynthetic reaction centre where the primary chemical reactions of photosynthesis occur. There is significant diversity in the types of light-harvesting proteins and the pigments they bind in various photosynthetic organisms. There are two major light-harvesting systems in cyanobacteria. Most "classical" cyanobacteria use external water-soluble bilin-bound protein complexes, phycobiliproteins (PBP), as their major light-harvesting system [4]; the new class of cyanobacteria, prochlorophytes (including *Prochlorococcus, Prochloron, Prochlorothrix*) uses integral membrane Chl-bound protein complexes, prochlorophyte chlorophyll *a*/*b* binding protein-Pcb, as their major antenna system [5].

Our previous work has identified a polypeptide with molecular mass of 35 kDa as a major light harvesting apoprotein in *Acaryochloris*, which binds primarily Chl *d* and a small amount of Chl *a* [6,7]. This antenna system is homologous to the Pcb-type light-harvesting protein and the amino acid sequence of the Pcb-type light-harvesting protein is homologous to the CP43/IsiA super-family of pigment-binding proteins with six-membrane-spanning helices [8]. There are two types of Pcb proteins in *Acaryochloris* [9,10], PcbA (now named accessory Chlorophyll Binding Protein, CBPII), associated with PS II reaction center (RC) core to form a giant supercomplex [11,12] and PcbC (now named as CBPI) associated with PS I, forming antenna-reaction centre supercomplexes [11,13].

The properties of ligands of various chlorophylls to the specific amino acids were studied using synthetic peptides (16 Aa) *in vitro*, which revealed that Chl *d* possesses similar coordination ligand properties to Chl *a*, and the Chl *d*-bound proteins are likely to bind

Abbreviations: BN, Blue native (native gel); CBP, accessory chlorophyll binding protein complex; Chl, chlorophyll; PAGE, polyacrylamide gel electrophoresis; *pcb*, prochlorophyte chlorophyll *a*/*b* binding protein; PCR, polymerase chain reaction; PS, photosystem

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chlorophyll *a* when chlorophyll *a* is available [14]. Other chlorophyll binding proteins also have shown the same binding flexibility of chlorophyll ligands. The isolated peridinin–chlorophyll–protein antenna system can recombine with a range of chlorophylls, peridinin and either Chl *a*, Chl *b*, Chl *d*, 3-acetyl-chlorophyll *a* or bacteriochlorophyll *a* [15]. The LHCaI apoprotein of *Porphyridium* has been shown to bind Chl *a*, Chl *b* or Chl *c* [16]. Sporlein and colleagues [17] have shown that BChl *a* in the accessory BChls and the bacterio-pheo's of bacterial reaction centers can be substituted by a number of other BChls. Chlorophyll *b* is synthesized by oxidation of the C-7 methyl group of chlorophyll *a* to a formyl group. This reaction is catalyzed by a Chlorophyll *a* oxygenase (CAO) enzyme [18]. Newly synthesized Chl *b* in a cyanobacterium by introducing the CAO enzyme can be functionally incorporated with photosynthetic systems [19,20]. These investigations have indicated that there seems to be no specific motif that is required to bind various types of chlorophylls. Furthermore, the genome of *Acaryochloris* reveals that most photosynthetic proteins that bind chlorophyll *d* in *Acaryochloris*, such as D1/D2/CP43/CP47 in photosystem II (PS II), and PsaA/PsaB in Photosystem I (PS I), show high homologies to Chl *a*-bound protein complexes from other cyanobacteria [21]. It is of interest to know how organisms evolved to utilize this longer-wavelength light energy. It is also interesting to know if the photosynthetic efficiency would be increased through the recombinant insertion of the intra-membrane light-harvesting complexes, CBPII-Chl *a* complexes.

Synechocystis sp. PCC 6803 is a well-studied cyanobacterium that has only Chl *a* and uses phycobilisomes (PBS) as its major antenna system. The integral membrane antenna proteins, CBPII from *Acaryochloris*, introduced into *Synechocystis* PCC 6803, are expected to bind Chl *a*, which is the only available chlorophyll in *Synechocystis* PCC 6803. This work reports the transformation of six-helix membrane antenna proteins into a foreign host and demonstrates its incorporation into the thylakoid membranes in the host strain, *Synechocystis* PCC 6803. Surprisingly, the incorporation of the integral chlorophyll-bound light-harvesting system changes the photosynthetic strategies for capturing the light, which provides insights into the regulatory mechanisms and the evolutionary significance of multiple alternatives of antenna systems in photosynthetic organisms.

2. Methods and materials

2.1. Culture conditions

Synechocystis sp. PCC 6803 wild type (WT) was cultivated at 25–27 °C under illumination of 10–30 μmol photons m^{-2} s^{-1} and continuous aeration in BG11 medium [22]. The transformed strain (*pcbA*$^{+}$) was cultured in the BG11 medium with additional 12 μg ml^{-1} kanamycin under the same culture conditions.

2.2. Recombinant construction

The *psbA3* (*sll1867*) upstream region (527 bp) was amplified by PCR with the pair of primers F1/ R1 (Table 1) from plasmid pWS19K (a kind gift from Dr. Wes Swingley). The *pcbA* gene was amplified from the vector pET32a(+)/*pcbA* (constructed by Dr. Chen) by PCR with the pair of primers F2/R2 (Fig. 1). The integrating PCR product (F1/R2) containing both *psbA3* upstream and *pcbA* gene was purified and digested with *Eco*RI/*Nde*I and then cloned into *Eco*RI/*Nde*I cut plasmid pWS19K (Fig. 1). The recombined vector pSW19K was transformed into *Synechocystis* PCC 6803 and these cells were grown on BG-11 agar plates for 18–20 h before they were transferred to plates supplemented with 12 μg mL^{-1} kanamycin for the selection of transformant colonies [23]. The segregation state of the transformants was monitored by PCR on isolated genomic DNA.

2.3. Isolation of thylakoid membranes

The cell pellets were washed twice by thylakoid buffer containing 20 mM MES, pH 6.4, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 20% glycerol (v/v), 1 mM PMSF and 5 mM benzamidine, and were ground in liquid nitrogen mixed with one volume of glass beads (Ø 0. 1 mm). After centrifugation (5000×*g*, 10 min) to remove cell debris, the supernatant was re-centrifuged (12,700×*g*, 20 min) to sediment the thylakoid membranes. The membranes were washed in the thylakoid buffer to remove the phycobiliproteins. The phycobiliprotein-free membranes were resuspended in the same buffer and stored at −70 °C for further investigation.

2.4. Separation of protein complexes by Blue Native (BN)-PAGE

A five μl protein sample of the thylakoid membranes was solubilised by adding 3 μl of thylakoid buffer supplemented with 10% dodecyl-β-D-maltoside (Sigma). The samples were incubated for 30 min at 4 °C, and unsolubilised material was removed by centrifugation at 12,700×*g*, 4 °C for 20 min. Half μl of the concentrated BN solubilisation buffer (10×) (100 mM BisTris, pH 7.0, 0. 5 M aminocaproic acid, 30% (w/v) sucrose) were added to 5 μl of solubilised samples and incubated for 5 min at 4 °C. The solubilised thylakoid membranes were separated by BN-PAGE at 4 °C as described in [24] using a 5–10% polyacrylamide gradient gel with some modifications. After electrophoresis, one gel was transferred onto a polyvinyl difluoride (PVDF) membrane for subsequent immunoblotting analysis. For the second dimension (2D) PAGE, the BN gel lanes (or bands) of interest were excised and incubated in a solution containing 25 mM Tris–HCl (pH 6.8), 3% (w/v) SDS and 5% (v/v) β-mercaptoethanol for 20 min, then transferred on top of a 10% polyacrylamide SDS-gel.

2.5. Electrophoresis and immunoblotting

The isolated thylakoid membranes were mixed with an equal volume of loading buffer (125 mM Tris–HCl pH 6.8, 4% (w/v) SDS, 20% glycerol, 10% (v/v) β-mercaptoethanol, 0.02% bromophenol blue), heated at 100 °C for 3 min and then electrophorised in 10% polyacrylamide SDS-gel at room temperature. The gels were visualized by staining with Coomassie Brilliant Blue R-250, unless they were to be used for immunoblotting. Protein separated by SDS-PAGE was transferred electrophoretically (90 V for 3 h) to PVDF membranes in the carbonate buffer pH 9.5 containing 20% (w/v) methanol. The membrane was blocked with 5% fat-free milkpowder in TBS (Tris buffered saline) and then incubated overnight at 4 °C with antibody

Table 1
The primer information.

Primers	Contents	Sequences
F1	*Nde*I + *psbA3* upstream	5′-ACTGAGTGCACCATATGTCAAGACCAGA-3′
R1	pWS19K	5′-AAGTTTGCATTATAATTCCTTATGTATTTGTCAATGT-3′
F2	*pcbA* (pET32a(+)/*pcbA*)	5′-ACAAATACATAAGGAATTATAATGCAAACTTATGGACAAACCG-3′
R2	*Eco*RI and His-tag	5′-CGGAATTCTTAGTGGTGGTGGTGGTGGTTTGCCTGAGGAGTTAG-3′
R3	*psbA3* downstream (pWS19K)	5′-CCAAATTCAAACCCTAGAGCATGC-3′

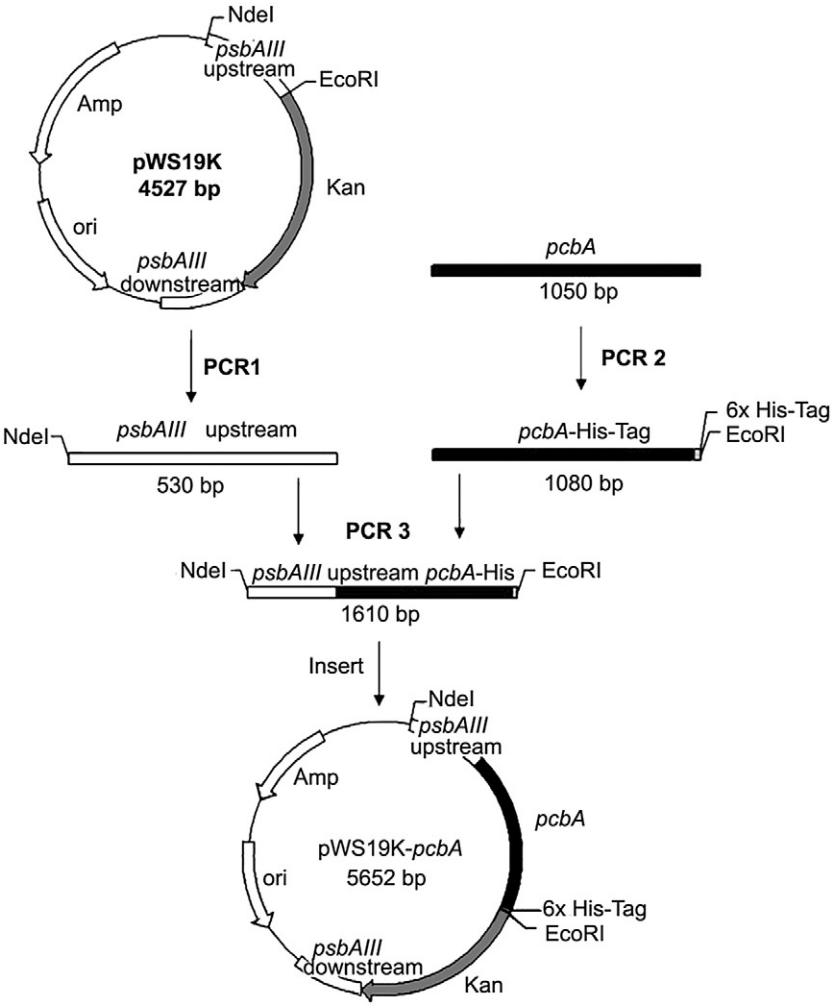

Fig. 1. Flow-chart for construction of recombinant *pcbA* expression vector.

(1:1000) diluted in TBS. Bound antibody was visualized by incubation using goat anti-mouse IgG conjugated with horseradish peroxidase (1:5000) for 30 min at room temperature followed by six washes in TBS-Tween buffer. The blot was developed using a BeyoECL Plus kit (Beyotime, China).

The green bands resolved in the native gel, containing CBPII protein–pigment complexes and PSII complexes, were excised and the protein complexes were eluted by electro-elution at 4 °C. The purified CBPII–pigment complexes were re-solubilised by 0.8% dodecyl-β-D-maltoside, 0.2% Triton and 0.1% Triton, respectively and loaded on a second native gel for further investigation.

2.6. Spectroscopic analysis

Pigment composition and concentration were analyzed using methanol extraction. In vivo absorbance spectra were recorded using a spectrophotometer (Shimadzu UV-2550, Japan) with a Taylor-sphere attachment (ISR-240A, Shimadzu, Japan) that reduces scattering. The spectra were normalized to the peak intensities of the chlorophyll Q_y transitions. The relative abundance of phycobiliproteins to Chl *a*, herein termed the PC index, was determined by using the maximum phycobilin absorbance (~624 nm) to the maximum Chl a absorbance (678 nm) ratio; i.e. (A624–A750)/(A678–A750). The relative abundance of carotenoid to chlorophyll, designated here the CX index, was calculated by using ratio of absorbance at 487 nm to the chlorophyll absorbance at 678 nm [25]. Fluorescence excitation/emission spectra were recorded at room temperature with a Varian Cary Eclipse Fluorescence spectrophotometer using diluted cells to 1 μg chlorophyll *a* per ml (Q_y absorbance was about 0.09) in fresh medium. To decrease the noise of fluorescence, the fluorescence spectra were obtained by accumulating 50 times with a 1 min interval time.

Low temperature fluorescence spectra were recorded with an OptistatDN cryostat (Oxford Instruments) attached to a Varian Cary Eclipse Fluorescence spectrophotometer. The cells were resuspended in a final concentration of 65% (v/v) of glycerol in seawater and frozen immediately using liquid nitrogen. The 77 K fluorescence spectra were smoothed using Origin 8 and normalized to the emission band at 682 nm (excitation at 435 nm) or normalized to the excitation band at ~630 nm (emission at 685 nm and 720 nm).

3. Results

3.1. Construction and expression of pcbA gene in Synechocystis sp.PCC6803

The plasmid pWS19K that contains the upstream and downstream regions of the *psbA3* gene (*sll1867*) from *Synechocystis* PCC 6803 and the *pcbA* gene (AM1_3654) of *Acaryochloris* with His-tag was constructed after the *psbA3* start code and a kanamycin resistance cartridge was linked after the *psbA3* gene (Fig. 1). The *pcbA* gene construction codes for a Chl *d*-binding protein (CBPII) under the control of the *Synechocystis sp*. PCC 6803 *psbA3* promoter. Two clones were confirmed to have the $pcbA^+$ genotype by PCR using primer pairs of F2/R2 and F1/R3 (Fig. 2). Fig. 2A demonstrates that the PCR

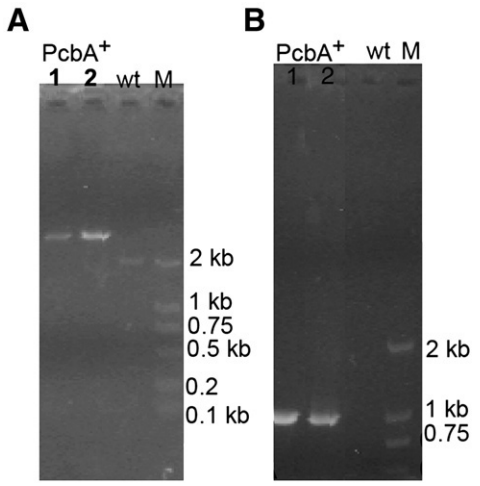

Fig. 2. Selection of the transformant strain. The genomic DNA of the transformants *pcbA*+ and the *wt* strains were isolated and the inserted fragments were tested by PCR using the different primers (Table 1). Lanes 1 and 2 are *pcbA*$^+$ strains and lane 0 is the control (wt strain). (A) The DNA fragments were generated using F1/R3 primers contains the *psbA3* upstream, *pcbA*, *kan* and *psbA3* downstream. (B) The DNA fragments were generated using F2/R2 primers contains the *pcbA* with His tag.

product of F1/R3 in the *pcbA*$^+$ strain is about 3. 0 kb, which is about 1. 0 kb bigger than the PCR fragment generated in the WT strain (without *pcbA*). This difference agrees with the size of the inserted *pcbA*. The expected PCR product of F2/R2 is about 1. 0 kb of *pcbA*$^+$ gene, but not in the control strain (Fig. 2B).

To characterise the expression of *pcbA* encoded foreign protein in the cell, SDS-PAGE and western blotting was performed on whole cell proteins and isolated thylakoid membranes. A band (~38 kDa) on the western blot (using His-tag antibody) confirmed the expression of CBPII in the *pcbA*$^+$ strain (Fig. supplementary).

3.2. The localization of the CBPII in Synechocystis

In order to determine the potential localization of expressed CBPII within the membranes and their relationship to other "native" chlorophyll-binding protein complexes, two-dimensional BN-PAGE/SDS-PAGE gels and immunoblotting were used. Four green bands were resolved from the BN-PAGE gel (Fig. 3A). The immuno-analysis of the BN-gel, using the His-tag antibody, showed that the third green band contained the expressed CBPII protein (Fig. 3B). Surprisingly, this green band also showed a positive reaction against a D1 antibody, a protein belonging to PSII, and indicates that it is also present (Fig. 3C). The co-existence of CBPII and PSII suggests that the transformed CBPII protein is associated with PSII in the transformant of *Synechocystis* PCC 6803.

2D-denaturing SDS-PAGE was used to analyse subunits of green bands of BN-PAGE. The number of subunits of PSI core complexes isolated from *Synechocystis* 6803 is 8–10 with apparent molecular masses of ~60, 18.5, 16, 15, 10.5, 9.5, and 6. 5 kDa [26]. Analysis of band 1 of the BN-PAGE gel indicated that the main subunits had

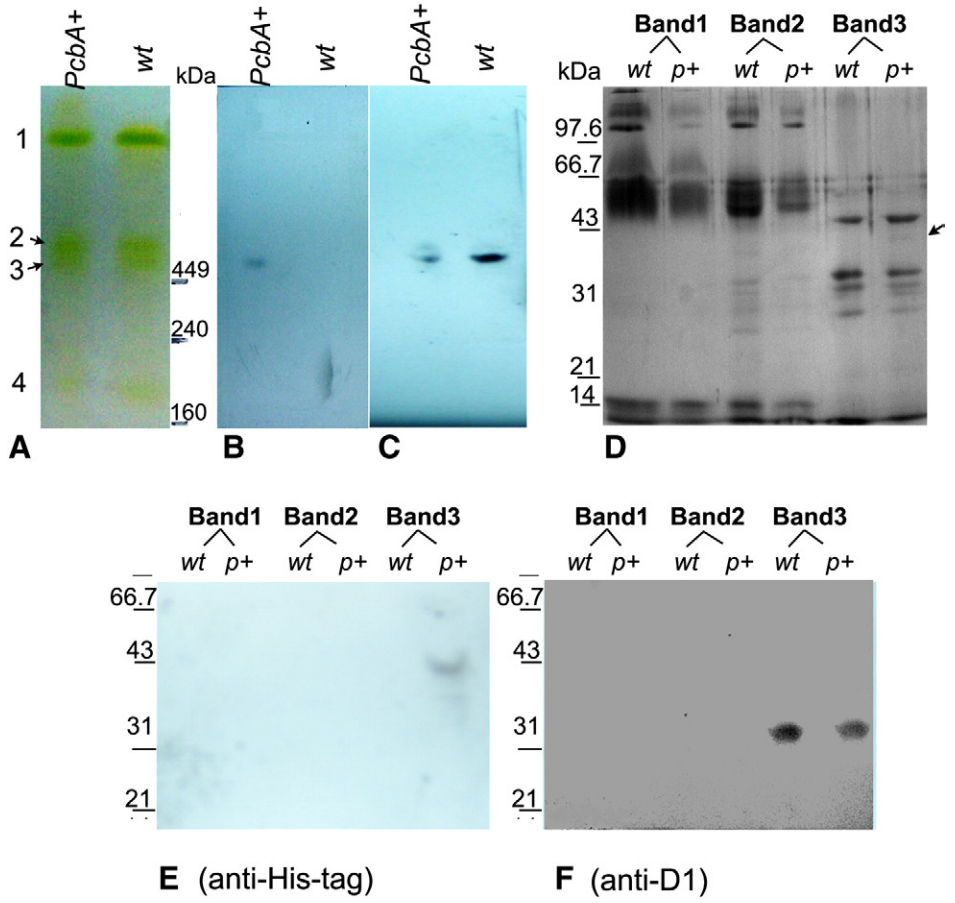

Fig. 3. Location of CBPII in thylakoid membranes by electrophoresis. Thylakoid membranes were isolated from *Synechocystis* PCC 6803 (*wt*) and *Synechocystis* PCC 6803 harbouring *pcbA* gene (*pcbA*$^+$). (A) Solubilised thylakoid membranes (Chl *a* = 0. 1 μg) were separated by BN-PAGE gel; (B) Immunoblotting analysis of native gel against the anti-His-tag antibody; (C) Immunoblotting analysis of native gel against anti-D1 antibody. Lane 1, *pcbA*+; lane 2, *wt*; (D) SDS-PAGE of green bands of BN-gel. Three green bands were cut out from BN gels respectively, extracted with 3% SDS and 5% β-mercaptoethanol in 25 mM Tris-HCl (pH 6.8), and resolved by SDS-PAGE. The arrow indicates the extra band existing in *pcbA*$^+$ sample; (E) Immunoblotting analysis of SDS-PAGE against the anti-His-tag antibody; (F) Immunoblotting analysis of SDS-PAGE against anti-D1 antibody. Band 1, the first green band of BN-gel; Band 2, the second green band and Band 3, the third green band. lanes 1,3,5, *wt*; lanes 2,4,6, *pcbA*$^+$(*P*$^+$).

molecular masses of ~60 kDa, 16 kDa, 15 kDa and 10. 5 kDa, which indicated that this green band is a PSI trimer with a molecular size of >750 kDa. The polypeptide composition of the second green band showed subunits originating from both PSI and PSII (core subunits CP47, CP43, D1 and D2 with apparent masses of 43, 37, 29, 33 kDa) [26]. According to the molecular size of ~600 kDa, this green band corresponds to the PSII dimer and PSI monomer. The molecular size of the third green band was about 500 kDa and the subunits of this green band were identified as belonging to the core of PSII. An extra band was identified in the *pcbA*$^+$ strain only (Fig. 3D, arrow). The immunoblots of SDS-PAGE use anti-His-tag and anti-D1 antibodies (Fig. 3E, F) confirmed that PSII and expressed CBPII pigment-bound–protein complexes co-exist in the third green band of native gel.

The second Native gel and immunoblotting against His-tag antibody are shown in Fig. 4. The different detergent solublisations produce different native pigment–protein complexes on the second native gel. Two green bands were resolved on the second native gel when the purified CBPII–pigment complexes were solubilised by 0.8% dodecyl-β-D-maltoside. The immunoblotting against the anti-His-tag antibody confirmed that both of them contain expressed CBPII and they are bound to Chl *a*. Interestingly, only one green band is resolved on the second native gel when the samples were solubilised by Triton (Fig. 4A). The immunoblotting showed one band as well (Fig. 4B).

3.3. Spectroscopic analyses of transformed Synechocystis PcbA$^+$

Shown in Fig. 5A are the room-temperature absorption spectra for *pcbA*$^+$ and WT cells lines normalized to the Q_y (678 nm) electronic transition of Chl *a*. The photosynthetic apparatus of both strains contain phycobilisomes since the electronic transition at ca. 624 nm originates from these peripheral antenna complexes. It is evident from the absorption spectra that the relative quantities of the chlorophyll, phycobilin and carotenoid pigments are not the same in these two strains (Fig. 5B). The *pcbA*$^+$ strain showed a decreased PC index (0.835 ± 0.017) compared to WT (1.377 ± 0.013). In contrast, the CX index showed the opposite trend, *pcbA*+ has higher CX index (1.18 ± 0.023). Furthermore, methanol extraction from whole cells (Fig. 5C) showed that *pcbA*+ contains a higher amount of carotenoid (see arrows). Fig. 4 indicates that the phycobilisome content in the transformant is decreased due to the presence of the extra integral antenna protein, CBPII.

The fluorescence excitation spectra of whole cells at room temperature were measured with the emission at 685 nm, PSII emission, or the emission at 720 nm, PSI emission (Fig. 6A). Both excitation spectra show a similar profile with a peak centered at 625 nm. The 625-nm peak is due to the absorbance of phycobiliproteins. This 625 nm signal showed much higher intensity in WT than this peak in the *pcbA*$^+$ sample. This also implies that the phycobiliprotein contributes to PSII in WT as a major antenna system, but not in *pcbA*$^+$. Three components, phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC), can be resolved in the 77 K fluorescence excitation spectra (Fig. 6C), although fluorescence excitation spectrum (emission at 685 nm) of the *pcbA*$^+$ sample showed a very small component of 650 nm compared to the WT sample.

The fluorescence spectral properties of intact cells at room temperature were measured with excitation at 435 nm, the excitation wavelength for Chl *a*. One main emission peak at 682 nm was observed from both strains (Fig. 6B). However, a shoulder at 655 nm was observed in WT, but not in the *pcbA*+ strain and an extra shoulder of ~705 nm in *pcbA*$^+$ was observed. In the 77 K fluorescence emission spectra of *pcbA*$^+$ (excitation at 435 nm), the bands centered at 720 nm is dominant when the spectra were normalised at 682 nm. The shoulder of 705 nm in the RT fluorescence emission spectrum (Fig. 6B) and the dominant peak centered at 720 nm in the 77 K emission spectrum (Fig. 6D) of *pcbA*$^+$ cells suggest that the newly introduced CBPII antenna complexes have induced a red-shifted peak of bound Chl *a*.

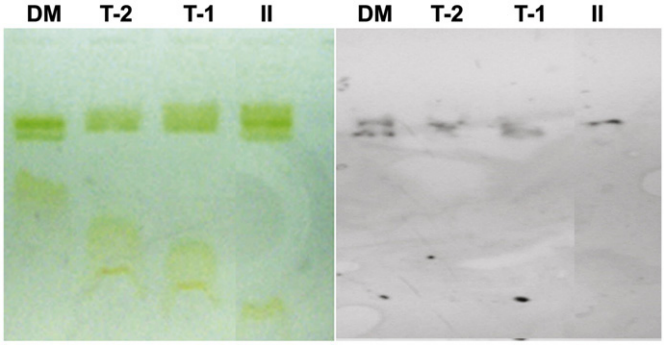

Fig. 4. The second native gel of CBPII–pigment complexes. The third green bands generated from first Native gel (Fig. 3A) were excised and re-collected using electro-elution. (A) the purified CBPII–pigment complexes were re-solubilised by 0.8% dodecyl-β-D-maltoside (lane 1, DM), 0.2% Triton (lane 2, T-2), 0.1 % Triton (lane 3, T-1), respectively. The lane 4 (II) is the purified third green band without further solubilisation, which contains PSII complexes (positive D1 antibody signal observed in immunoblotting) and CBPII complexes (His-tag antibody positive); (B) the immunoblotting analysis of the second native gel against anti-His-tag antibody.

4. Discussion

The flexibility of light-harvesting proteins in the incorporation of different chlorophylls allows photosynthetic organisms to take advantage of a particular light-harvesting Chl that they are able to synthesize. Incorporation of Chl *d*-binding light-harvesting proteins into *Synechocystis* and the transformant *pcbA*$^+$ is merely a consequence of expression and utilization of the foreign protein by "feeding" (binding) it with Chl *a*, the only chlorophyll it can synthesize.

Detergent extraction and non-denaturing BN-PAGE, coupled with denaturing SDS-PAGE and immunoblotting are accepted approaches to separate native protein complexes and identify their subunits. These methods have been used successfully to study membrane-bound protein complexes [26,27]. In this work, we have demonstrated that the BN-PAGE of isolated thylakoid membranes contain similar photosynthetic protein complexes in *pcbA*+ and WT strains (Fig. 3A). Immunoblotting confirmed that the CBPII was successfully transformed and expressed in the host *Synechocystis* cells and located in the thylakoid membranes, but bound with Chl *a*.

The protein CBPII in *Acaryochloris* is encoded by the *pcbA* gene (AM1_3654) and has been shown to function as an antenna protein complex associated with PSII [12]. The transformed CBPII in the *pcbA*$^+$ strain showed that it is associated exclusively with PSII (Fig. 3). This supports the idea that the integral light-harvesting systems that are specific antennae for PSII are different from the antennae for PSI [10,11].

Two pools of Chl *a* were observed in *pcbA*+, a Chl *a* emission at 682 nm and another at ~705 nm, which confirmed different environments of antennae in the transformant *pcbA*+. The new pool of Chl *a* at 705 nm in RT emission spectrum and 723 nm in 77 K emission spectrum was only found in *pcbA*+. However, it is uncertain whether this component is attributable only to the transformed light-harvesting protein, CBPII. Protein may play an important role for optical properties of bound pigments. An iron-stress-induce A protein (IsiA, newly named as CBPIII type proteins) have been found to form an 18-mer ring around the PSI trimer complex [28,29]. The fluorescence emission spectrum of isolated Chl *a*-IsiA complexes showed peak of 685 nm at 77 K (excitation at 435 nm) [30], CBPII protein has similar structure as isiA, but bound Chl *d* in *Acaryochloris*. The Chl-binding sites in both proteins are conserved, although they are only shared about 68% homologous sites [9]. The isolated native CBPII protein bound Chl *d* showed fluorescence emission peak of

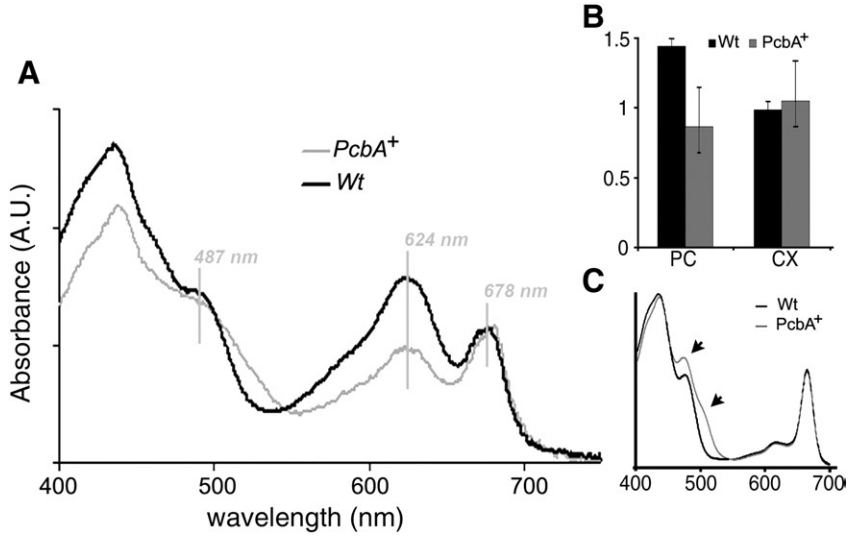

Fig. 5. Characteristic absorption spectral analyses. (A) Room temperature absorption spectra of *Synechocystis* strains (whole cells). (B) PC index and CX index are calculated based on 4 individual cultures and 16 repeats. Error bars are standard deviation of 16 repeats. (C) Spectral comparison of total pigment extraction using 100% methanol. Arrows indicate the differences between two strains. Black lines represent *Synechocystis wt* strain; grey lines represent *Synechocystis pcbA*$^+$ strain.

704 nm at 77 K (excitation at 398 nm), which is similar to its absorbance peak of 703 nm at room temperature [12]. The red shoulder of 705 nm in the *pcbA*$^+$ strain at room temperature (Fig. 6B) and emission peak of 720 nm at low temperature (Fig. 6D), suggested that expressed CBPII caused the red-shifting of the bound Chl *a*, which confirmed that different protein environments can change the optical

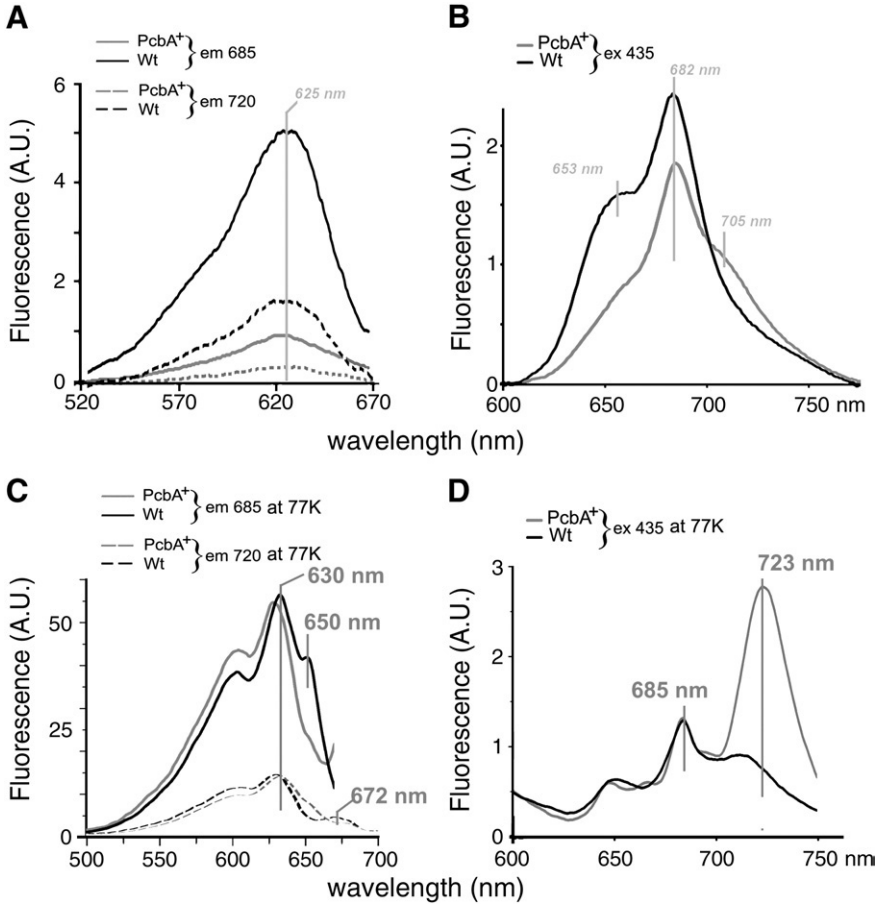

Fig. 6. fluorescence spectral analyses. (A) Room temperature fluorescence excitation spectra of *Synechocystis* strains. The spectra are averages of 15 runs with one minute interval time. Solid lines are emission at 685 nm; dashed lines are emission at 720 nm. (B) Room temperature fluorescence emission spectra of *Synechocystis* strains. The spectra were recorded using excitation wavelength of 435 nm and presented as average of 50 runs with one minute interval time. (C) 77 K fluorescence excitation spectra of *Synechocystis* strains. The spectra were recorded using emission wavelengths of 685 nm and 720 nm, respectively, and presented as average of 50 runs with one minute interval time. The spectra were normalized at the peak of 630 nm. (D) 77 K fluorescence emission spectra of *Synechocystis* strains. The spectra were recorded using excitation wavelength of 435 nm and presented as average of 50 runs with one minute interval time. The spectra were normalized at the peak of 682 nm. Black lines represent *Synechocystis wt* strain; grey lines represent *Synechocystis pcbA*$^+$ strain.

properties of the bound chlorophylls. Further investigation of isolated pigment–protein complexes and in vitro reconstitution will be needed to resolve this issue.

The steady-state excitation spectra showed clearly that almost no excitation peak of PBS can be observed in *pcbA*+ when the emission wavelengths was chosen at 685 nm (for PSII) and at 720 nm (for PSI). The introduced integral light-harvesting protein (CBPII) apparently competes with the existing external antenna system, the phycobiliproteins. The transformed CBPII protein in *pcbA*+ has 40% less PBS according to the PC index (Fig. 5B). The missing emission shoulder of allophycocyanin (at 655 nm) and the extra shoulder of 705 nm in fluorescence emission spectra of *pcbA*+ provide direct evidence that incorporation of CBPII into *Synechocystis* has changed the cell's light-harvesting strategies, with downregulation of phycobiliproteins and with upregulation of the integral membrane-bound antenna system.

The BN-PAGE analysis showed the green band of CBPII proteins, which indicates that chlorophylls are bound to the CBPII protein. Chlorophyll *a* is the only available chlorophyll in *Synechocystis*. The incorporated CBPII protein in *Synechocystis*, strain *pcbA*$^+$, caused changes of its pigment composition, decreased PC index and increased CX index (Fig. 5B). Carotenoids are essential accessory pigment for stabilization of chlorophyll-binding protein complexes. The PSII crystal structure reveals that there are 5 carotenoids bound to CP43 [31]. CBPII belongs to the CP43 super-family with the conserved pigment ligand sites that bind chlorophylls and carotenoids [8,9]. The carotenoids function as accessory pigments to stabilise CP43 structure, to transfer the energy to other molecules or to accept excess excitation energy in the photosynthetic systems as an energy quencher. However, no carotenoids are required in the architecture of the phycobiliprotein external antenna system. The observed higher carotenoid content in the *pcbA*$^+$ transformant may be required to meet the needs for the incorporated CBPII protein. The carotenoid also may play roles in photoprotection/energy dissipation.

Phycobiliproteins exist only in cyanobacteria and limited species of algae, such as glaucophytes, cryptophytes and red algae. The bound phycobilins allow the photosynthetic organisms to extend their light absorbing into the “green window” of chlorophylls (500–650 nm, although the supramolecular architecture of phycobiliprotein complexes is “expensive” for the photosynthetic organisms according to their higher protein content, the low energy efficiency, and the higher biosynthetic cost for phycobilins [32].

On the other hand, introduced CBPII antenna system allow *Synechosystis pcbA*$^+$ using Chl *a* as its functional antenna chromophore with the absorbing light wavelength of 675–700 nm, Φ_e can reach to 99–103% efficiency if the energy is transferred to the PSII RC. In general, cyanobacteria either use water-soluble phycobiliproteins or the membrane-bound chlorophyll-binding protein-complexes (CBP) as their distal antennae [33,34]. *Synechocystis* PCC 6803 uses phycobilisomes as its major light-harvesting system, but *Acaryochloris* uses Chl *d*-binding protein complexes, CBPs, as its major light-harvesting system [6]. It is worth noting that *Acaryochloris* MBIC 11017 has primitive degraded phycobiliproteins, which do not form typical phycobilisomes [35]. These phycobiliproteins act as an additional minor antenna system [35–37]. The regulatory relationship of two antenna systems in *Acaryochloris* has been investigated and the competitive regulation between them revealed [38]. There are two hypothetical mechanisms: competition is based on the competition of pigment biosynthesis [32] or based on the spatial capability, i.e. the extra integral antenna system inhibits the spatial connection between phycobiliprotein and PSII.

The six-helix intra-membrane accessory chlorophyll-binding protein complexes are only found in oxygenic photosynthetic bacteria (cyanobacteria) and it is believed that they are a “late” evolutionary phenomenon, with an origin from CP43 [39]. Although it is clear that lateral gene transfer is a mechanism of various antenna apparatus development in all oxygenic photosynthetic bacteria, the evolutionary relatedness between phycobilinprotein antenna systems and chlorophyll-bound antenna systems still remains uncertain [10,34,39,40].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2009.10.006.

References

[1] H. Miyashita, H. Ikemoto, N. Kurano, K. Adachi, M. Chihara, S. Miyachi, Chlorophyll *d* as a major pigment, Nature 383 (1996) 402.
[2] H. Miyashita, K. Adachi, N. Kurano, H. Ikemoto, M. Chihara, S. Miyachi, Pigment composition of a novel oxygenic photosynthetic prokaryote containing chlorophyll *d* as the major chlorophyll, Plant Cell Physiol. 38 (1997) 274–281.
[3] M. Kühl, M. Chen, P. Ralph, U. Schreiber, A.W.D. Larkum, A niche for cyanobacteria containing chlorophyll *d*, Nature 433 (2005) 820.
[4] A.R. Grossman, D. Bhaya, K.E. Apt, D.M. Kehoe, Light-harvesting complexes in oxygenic photosynthesis: diversity, control and evolution, Annu. Rev. Genet 29 (1995) 231–287.
[5] H.C.P. Matthijs, G.W.M. van der Staay, L.R. Mur, Prochlorophytes: the other cyannobacteria? in: D.A. Bryant (Ed.), The Molecular Biology of Cyanobacteria, Kluwer Academic publisher, Dordrecht, 1995, pp. 49–64.
[6] M. Chen, R.G. Quinnell, A.W.D. Larkum, Chlorophyll *d* as the major photopigment in *Acaryochloris marina*, J. Porph. Phthalo. 6 (2002) 763–773.
[7] M. Chen, R.G. Quinnell, A.W.D. Larkum, The major light-harvesting pigment protein of *Acaryochloris marina*, FEBS Lett. 514 (2002) 149–152.
[8] J. La Roche, G.W.M. van der Staay, F. Partensky, A. Ducret, R. Aebersold, R. Li, S.S. Golden, R.G. Hiller, P.M. Wrench, A.W.D. Larkum, B.R. Green, Independent evolution of the prochlorophyte and green plant chlorophyll *a*/*b* light-harvesting proteins, Proc. Nat. Acad. Sci. U.S.A. 93 (1996) 15244–15248.
[9] M. Chen, R.G. Hiller, C.J. Howe, A.W.D. Larkum, Unique origin and lateral transfer of prokaryotic chlorophyll-*b* and chlorophyll *d* light-harvesting systems, Mol. Biol. Evol. 22 (2005) 21–28.
[10] M. Chen, T.S. Bibby, Photosynthetic apparatus of antenna-reaction centres supercomplexes in oxyphotobacteria: insight through significance of Pcb/IsiA proteins, Photosynth. Res. 86 (2005) 165–173.
[11] M. Chen, Y. Zhang, R.E. Blankenship, Nomenclature for membrane bound light harvesting complexes of cyanobacteria, Photosynth. Res. 95 (2008) 147–154.
[12] M. Chen, T.S. Bibby, J. Nield, A.W.D. Larkum, J. Barber, Structure of a large photosystem II supercomplex from *Acaryochloris marina*, FEBS Lett. 579 (2005) 1306–1310.
[13] M. Chen, T.S. Bibby, J. Nield, A.W.D. Larkum, J. Barber, Iron effects on function and localisation of antenna system binding with Chl *d*, Biochim. Biophys. Acta 1708 (2005) 367–374.
[14] M. Chen, L. Eggink, J.K. Hoober, A.W.D. Larkum, Influence of structure on binding of chlorophylls to peptide ligands, J. Amer. Chem. Soc. 127 (2005) 2052–2053.
[15] R.P. Ilagan, T.W. Chapp, R.G. Hiller, F.P. Sharples, T. Polívka, H.A. Frank, Optical spectroscopic studies of light-harvesting by pigment-reconstituted peridinin–chlorophyll–proteins at cryogenic temperatures, Photosynth. Res. 89 (2006) 1–13.
[16] B. Grabowski, S. Tan, F.X. Cunningham Jr, E. Gantt, Characterization of the *Porphyridium cruentum* Chl *a*-binding LHC by in vitro reconstitution: LHCaR1 binds 8 Chl *a* molecules and proportionately more carotenoids than CAB proteins, Photosynth. Res. 63 (2000) 85–96.
[17] S. Sporlein, W. Zinth, M. Meyer, H. Scheer, J. Wachtveitl, Primary electron transfer in modified bacterial reaction centers: optimization of the first events in photosynthesis, Chem. Phys. Lett. 322 (2000) 454–464.
[18] A. Tanaka, H. Ito, R. Tanaka, N.K. Tanaka, K. Yoshida, K. Okada, Chlorophyll *a* oxygenase (CAO) is involved in chlorophyll *b* formation from chlorophyll *a*, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 12719–12723.
[19] H. Xu, D. Vavilin, W. Vermaas, Chlorophyll *b* can serve as the major pigment in functional photosystem II complexes of cyanobacteria, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 14168–14173.
[20] S. Satoh, M. Ikeuchi, M. Mimuro, A. Tanaka, Chlorophyll *b* expressed in cyanobacteria functions as a light-harvesting antenna in photosystem I through flexibility of the proteins, J. Biol. Chem. 276 (2001) 4293–4297.
[21] W.D. Swingley, M. Chen, P.C. Cheung, AL. Conrad, L.C. Dejesa, J. Hao, B.M. Honchak, L.E. Karbach, A. Kurdoglu, S. Lahiri, S.D. Mastrian, H. Miyashita, L. Page, P.

Ramakrishna, S. Satoh, W.M. Sattley, Y. Shimada, H.L. Taylor, T. Tomo, T. Tsuchiya, Z.T. Wang, J. Raymond, M. Mimuro, R.E. Blankenship, J.W. Touchman, Niche adaptation and genome expansion in the chlorophyll d-producing cyanobacterium *Acaryochloris marina*, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 2005–2510.

[22] M.M. Allen, Methods for cyanophyceae, in: J.R. Stein (Ed.), Handbook of Phycological Methods: Culture Methods and Growth Measurements, Cambridge University Press, Cambridge, UK, 1973, pp. 127–138.

[23] W.F.J. Vermaas, J.G.K. Williams, C.J. Arntzen, Sequencing and modification of psbB, the gene encoding the CP-47 protein of photosystem II, in the cyanobacterium *Synechocystis* 6803, Plant Mol. Biol. 8 (1987) 317–326.

[24] H. Schagger, G. von Jagow, Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form, Anal. Biochem. 199 (1991) 223–231.

[25] R.S. Gloag, R.J. Ritchie, M. Chen, A.W.D. Larkum, R.G. Quinnell, Chromatic photoacclimation, photosynthetic electron transport and oxygen evolution in the chlorophyll d-containing oxyphotobacterium *Acaryochloris marina*, Biochim. Biophys. Acta 1767 (2007) 127–135.

[26] E.M. Aro, M. Suorsa, A. Rokka, Y. Allahverdiyeva, V. Paakkarinen, A. Saleem, N. Battchikova, E. Rintamaki, Dynamics of photosystem II: A proteomic approach to thykakoid protein complexes, J. Exp. Bot. 56 (2005) 347–356.

[27] E. Thidholm, V. Lindstrom, C. Tissier, C. Robinson, W.P. Schroder, C. Funk, Novel approach reveals localization and assembly pathway of the PsbS and PsbW proteins into the photosystem II dimmer, FEBS Lett. 513 (2002) 217–222.

[28] T.S. Bibby, J. Nield, J. Barber, Iron deficiency induces the formation of an antenna ring around trimeric photosystem I in cyanobacteria, Nature 412 (2001) 743–745.

[29] E.J. Boekema, A. Hifney, A.E. Yakushevska, M. Piotrowski, W. Keegstra, S. Berry, K.-P. Michel, E.K. Pistorius, J. Kruip, A giant chlorophyll-protein complex induced by iron-deficiency in cyanobacteria, Nature 412 (2001) 745–748.

[30] T.S. Bibby, J. Nield, J. Barber, Three-dimensional model and characterization of the iron stress-induced CP43′-photosystem I supercomplex isolated from the cyanobacterium *Synechocystis* PCC 6803, J. Biol. Chem. 276 (2001) 43246–43252.

[31] A. Zouni, H.T. Witt, J. Kern, P. Fromme, N. Krauss, W. Saenger, P. Orth, Crystal structure of photosystem II from Synechococcus elongates at 3. 8 Å resolution, Nature 409 (2001) 739–743.

[32] H. Scheer, The pigments, in: B.R. Green, W.W. Parson (Eds.), Light-Arvesting Antennas in Photosynthesis, Springer, 2003, pp. 29–81.

[33] T.S. Bibby, Y. Zhang, M. Chen, Biogeography of photosynthetic light-harvesting genes in marine phytoplankton, PLoS ONE 4 (2009) e4601 doi:10.1371.

[34] C.S. Ting, G. Rocap, J. King, S.W. Chisholm, Cyanobacterial photosynthesis in the oceans: the origins and significance of divergent light-harvesting strategies, Trends Microbiol. 10 (2002) 134–142.

[35] Q. Hu, J. Marquardt, I. Iwasaki, H. Miyashita, N. Kurano, E. Morschel, S. Miyachi, Molecular structure, localization and function of biliproteins in the chlorophyll *a*/*d* containing oxygenic photosynthetic prokaryote *Acaryochloris marina*, Biochim. Biophys. Acta 1412 (1999) 250–261.

[36] J. Marquardt, S. Horst, H. Miyashita, S. Miyachi, E. Moerschel, Isolation and characterization of biliprotein aggregates from *Acaryochloris marina*, a *Prochloron*-like prokaryote containing mainly chlorophyll *d*, FEBS Lett. 410 (1997) 428–432.

[37] Z. Petrasek, F.J. Schmitt, C. Theiss, J. Huyer, M. Chen, A. Larkum, H.J. Eichler, K. Kemnitz, H.J. Eckert, Excitation energy transfer from phycobiliprotein to chlorophyll *d* in intact cells of *Acaryochloris marina* studied by time- and wavelength-resolved fluorescence spectroscopy, Photochem. Photobiol. Sci. 12 (2005) 1016–1022.

[38] M. Chen, K. Donohoe, B. Crossett, M. Schliep, T. Larkum, Molecular bases of antenna system adaptation in a Chl *d*-containing organism, in: J.F. Allen, O. Osmond, J.H. Golbeck, E. Gantt (Eds.), Photosynthesis: Energy from the Sun, Springer, 2008, pp. 243–246.

[39] Y. Zhang, M. Chen, BB. Zhou, LS. Jermiin, AWD. Larkum, Evolution of the inner light-harvesting antenna protein family of cyanobacteria, algae, and plants, J. Mol. Evol. 64 (2007) 321–331.

[40] A. Tomitani, K. Okada, H. Miyashita, HCP. Matthijs, T. Ohno, A. Tanaka, Chlorophyll b and phycobilins in the common ancestor of cyanobacteria and chloroplasts, Nature 400 (1999) 159–162.