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# Analysis of photosynthetic complexes from a cyanobacterial *ycf37* mutant

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**Abstract**

The Ycf37 protein has been suggested to be involved in the biogenesis and/or stability of the cyanobacterial photosystem I (PSI) [A. Wilde, K. Lünser, F. Ossenbühl, J. Nickelsen, T. Börner, Characterization of the cyanobacterial *ycf37*: mutation decreases the photosystem I content, Biochem. J. 357 (2001) 211–216]. With Ycf37 specific antibodies, we analyzed the localization of Ycf37 within the thylakoid membranes of the cyanobacterium *Synechocystis* sp. PCC 6803. Inspection of a sucrose gradient profile indicated that small amounts of Ycf37 co-fractionated with monomeric photosynthetic complexes, but not with trimeric PSI. Isolating 3xFLAG epitope-tagged Ycf37 by affinity-tag purification rendered several PSI subunits that specifically co-precipitated with this protein. Blue-native PAGE newly revealed two monomeric PSI complexes (PSI and PSI*) in wild-type thylakoids. The lower amount of PsaK present in PSI* may explain its higher electrophoretic mobility. PSI* was more prominent in high-light grown cells and interestingly proved absent in the Δ*ycf37* mutant. PSI* appeared again when the mutant was complemented in trans with the wild-type *ycf37* gene. In the Δ*ycf37* mutant the amount of trimeric PSI complexes was reduced to about 70% of the wild-type level with no significant changes in photochemical activity and subunit composition of the remaining photosystems. Our results indicate that Ycf37 plays a specific role in the preservation of PSI* and the biogenesis of PSI trimers.


*Keywords:* Chloroplast open reading frame; Photosystem I; *Synechocystis* sp. PCC 6803; Thylakoid

## 1. Introduction

Chloroplasts which have arisen from a cyanobacterial ancestor [1] maintained an independent circular genome encoding gene products which are important for primary and secondary processes of photosynthesis. Most of the remaining genes are required for transcription, translation or replication of the plastid DNA. In addition, highly conserved *ycfs* (hypothetical chloroplast open reading frames) of initially unknown function have been identified in the chloroplast genomes of higher plants. Other *ycfs* are absent from higher plant chloroplast genomes but still present in the plastid genomes of algae and in the genomes of cyanobacteria. These genes may have been transferred from the chloroplast to the nuclear DNA in evolution. Most of the *ycfs*-mutants induced in tobacco, *Chlamydomonas reinhardtii* and in the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) have impaired photosynthetic functions. For example *ycf3* and *ycf4* encode assembling and/or stabilizing factors for photosystem (PS) I [2–4], whereas *ycf5* seems to be involved in cytochrome *c* biogenesis [5]. Ycf7 and Ycf9 were shown to be subunits of the cytochrome $b_6f$ and PSII complexes, respectively [6,7].

The open reading frame *ycf37* is conserved in the algal chloroplast genomes of *Cyanophora paradoxa* (accession no. NC_001675), *Cyanidium caldarium* (accession no. NC_ 001840), *Porphyra purpurea* [8] and *Guillardia theta* [9], but cannot be identified in the plastome of higher plants and green algae. However, a nuclear encoded *Arabidopsis thaliana* gene product (At1g22700) shows similarity to the *Synechocystis* 6803 Ycf37 protein (35% identity, 62% similarity and identity) [10]. Insertional inactivation of *ycf37* in *Synechocystis* 6803 resulted

*Abbreviations:* BN, blue-native; Chl, chlorophyll a; β-DM, n-dodecyl-β-D-maltoside; PCC, Pasteur Culture Collection; PMSF, phenylmethanesulfonyl fluoride; PSI, photosystem I; PSII, photosystem II; TPR, tetratrico-peptide repeat

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in a diminished PSI content but did not significantly influence photosynthetic growth under standard conditions [10].

The Ycf37 protein contains three tetratrico-peptide repeat (TPR) motifs. TPR-proteins are found in almost all organisms and comprise a variety of functions, typically by mediating protein–protein interactions. The specificity of these interactions with target proteins has been well established for several proteins [11]. Structurally, a TPR consists of a degenerate motif of 34 amino acids forming two short amphipathic α-helices. These typically occur in tandem arrays of 3–16 motifs in parallel orientation. Interestingly, Ycf3 which is posttranslationally involved in PSI biogenesis also contains three TPR motifs, like Ycf37 [3,10]. Naver et al. showed that in *Chlamydomonas reinhardtii* Ycf3 interacts with the PSI subunits PsaA and PsaD [2]. Deletion of *ycf3* in *Chlamydomonas* [3], tobacco [4] and *Synechocystis* 6803 [12] resulted in a complete loss of functional PSI. In Δ*ycf37* mutants of *Synechocystis* 6803, however, PSI remains active but its abundance is decreased to about 70% of the wild-type level [10]. In recent years, several other factors involved in the biogenesis of PSI have been identified in eukaryotic as well as prokaryotic organisms [13–16]. Nevertheless, the detailed mechanism of assembly of the photosynthetic apparatus and the molecular function of the various assembling factors remain poorly understood. The identification of the loci where these putative assembling factors operate should greatly increase our understanding of biogenesis of photosynthetic protein complexes.

In accordance with the predicted single membrane-spanning region in the Ycf37 protein we showed that it is mainly localized in the thylakoid membrane [17]. In order to identify the site of Ycf37 action, we have studied the localization of the protein in the thylakoid membrane and its possible interaction with photosynthetic membrane complexes by using affinity-purification of 3xFLAG epitope-tagged Ycf37. Furthermore, the membrane protein complexes of the wild-type and Δ*ycf37* mutant strains were subjected to 2-D blue-native (BN)/SDS-PAGE analysis. We have identified a PSI monomer complex in wild-type thylakoids that was absent in the Δ*ycf37* mutant.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

Liquid cultures of *Synechocystis* 6803 wild-type (this strain originated from the laboratory of S. Shestakov, Moscow State University) and mutant strains were grown at 30 °C in BG-11 medium [18] under continuous illumination with white light of 50 μmol photons $m^{-2}$ $s^{-1}$ and bubbled with air. The medium for the Δ*ycf37* mutant strain [10] was supplemented with 7 μg $ml^{-1}$ chloramphenicol.

### 2.2. Mutagenesis

For reversion of the Δ*ycf37* mutants to the wild-type phenotype, a 1061-bp fragment bearing the *ycf37* gene together with upstream and downstream sequences was amplified using the primers 5′-TACCTGGGAGGGGAAGAAAG-3′ and 5′-CCAATTTTTCCTGGTCATCC-3′. The fragment was subcloned into the pCR2.1 TOPO vector (Invitrogen), then excised by *Eco*RI digestion and inserted into the chloramphenicol-resistance gene cartridge of the pVZ321 vector [19]. The pVZ*ycf37* vector harbouring the *ycf37* gene flanked by its promoter and terminator regions was transferred to Δ*ycf37* cells by conjugation according to [19] and exconjugants were selected on BG-11 agar plates containing 40 μg $ml^{-1}$ kanamycin.

### 2.3. Purification of tagged Ycf37 from Synechocystis 6803

The pCR2.1 vector bearing the *ycf37* gene together with upstream and downstream sequences (see Section 2.2) was used to create a *Nde*I site at the 3′ end of the *ycf37* coding region to facilitate ligation of a tag-encoding DNA-fragment. The *Nde*I site was inserted using the QuickChange Mutagenesis Kit (Stratagene) and primers 5′-CCAATTCCTTGAAAAAACGGCATATGGAGACTTAGAATAGATTGGG-3′, 5′-CCCAATCTATTCTAAGTCTCCATATGCCGTTTTTTCAAGGAATTGG-3′ thereby changing C-terminal L172 to H and V173 to M. Two complementary oligonucleotides encoding the 3xFLAG peptide (Sigma) were hybridized, thus creating 5′overhangs that were ligated into the *Nde*I site two amino acids upstream of the *ycf37* stop codon. The resultant construct was verified by sequencing. Restriction with *Eco*RI yielded a DNA fragment encoding the FLAG-tagged Ycf37 protein with its own promoter and terminator sequences that was then ligated into pVZ321. This vector (pVZ*ycf37*FLAG) was transferred to Δ*ycf37* mutant cells by conjugation as described above. Protein extracts of mutant cells (0.5 mg chlorophyll (Chl)) expressing the FLAG-tagged Ycf37 protein (as a control extracts of a Δ*ycf37* mutant expressing *ycf37* without the FLAG-tag in trans were used) were solubilized 10 min with 10 mg n-dodecyl-β-D-maltoside (β-DM) at 4 °C. Non-solubilized material was separated by centrifugation (13,000 × *g*, 30 min, 4 °C) and the supernatant has been incubated with 200 μl of anti-FLAG M2 agarose (Sigma) in thylakoid buffer containing 150 mM NaCl and 0.04% β-DM for 2 h at 4 °C. The resin was then washed 5 times with 10 column volumes of incubation buffer, and proteins were eluted three times with 100 μg $ml^{-1}$ of 3xFLAG peptide in the same buffer. Eluates were concentrated by using Vivaspin 500 tubes (Vivascience, Hannover, Germany).

### 2.4. Preparation of thylakoid membranes

Cultures of *Synechocystis* 6803 wild type and Δ*ycf37* in the logarithmic growth phase were harvested by centrifugation at 4000 × *g* for 10 min. The pellet was resuspended in three volumes of thylakoid buffer (50 mM HEPES/NaOH, pH 7.0, 5 mM $MgCl_2$, 25 mM $CaCl_2$, 10% (v/v) glycerol). The cells were disrupted in a bead beater (Hamilton Beach) using glass beads of 0.25–0.5 mm. Glass beads and unbroken cells were removed by centrifugation for 5 min at 4000×*g* and the supernatant containing the thylakoid membranes was centrifuged for 30 min at 15,000 × *g*. Thylakoid membranes were resuspended in thylakoid buffer to a concentration of about 1 mg Chl a $ml^{-1}$ corresponding to about 20 mg protein $ml^{-1}$, respectively. Isolated membranes were highly enriched with thylakoids but also contained fragments of the plasma membrane. Aliquots were stored at −80 °C.

### 2.5. Sucrose density gradients and separation of PSI and PSII monomers

The thylakoid membranes were diluted in thylakoid buffer to a protein concentration of 10 mg $ml^{-1}$ and solubilized with β-DM at a detergent-to-protein ratio of 2:1 in the dark at 4 °C for 10 min. Non-solubilized material was removed by centrifugation at 30,000 × *g* for 30 min. The supernatant was loaded onto a linear sucrose density gradient (10–30% (w/v) sucrose) in thylakoid buffer without glycerol but containing 0.04% (w/v) β-DM. Ultracentrifugation was performed in a SW28 rotor for 16 h at 140,000 × *g* and 4 °C (Beckman L70 ultracentrifuge). Fractions obtained from the sucrose gradient were investigated by SDS-PAGE and immunoblot analysis. PSI and PSII monomers were further separated on a HPLC anion-exchange column (Resource Q, Pharmacia Biotech, Uppsala Sweden) as described [20] applying a nonlinear $MgSO_4$ gradient. Peak fractions of PSI and PSII were collected, concentrated and desalted by using Vivaspin 4 tubes (Vivascience, Hannover, Germany).

### 2.6. SDS-PAGE and immunoblot

Proteins were separated by SDS-PAGE [21]. Acrylamide concentrations were 4.5% for the stacking and 12.5% or 15% (w/v) for the resolving gels.

Tricine–SDS-PAGE with 6 M urea was performed as described [22]. Protein concentrations were estimated according to Lowry et al. [23]. After electrophoresis, proteins were electrophoretically transferred onto nitrocellulose membranes and immunodecorated with specific antibodies. Immunolabelled bands were visualized with a goat anti-rabbit IgG-peroxidase conjugate and SuperSignal West Pico as chemiluminescent substrate (Pierce). Signal intensities were quantified densitometrically using the LumiImager F1 (Roche Diagnostics) and the Quantity One 4.1.1 software (Bio-Rad Laboratories).

### 2.7. BN-PAGE

Thylakoid membranes (about 500 μg protein) were sedimented by centrifugation (20 min, 15,000 × *g*, 4 °C) and resuspended in 50 μl of ACA buffer (50 mM Bis-Tris/HCl, pH 7.0, 750 mM ε-amino-n-caproic acid, 0.5 mM EDTA). Membrane proteins were solubilized by the addition of 10 μl freshly prepared 10% (w/v) β-DM solution. After centrifugation (15,000 × *g*, 30 min), the supernatants were supplemented with 10 μl of a Coomassie Blue solution (5% (w/v) Serva Blue G, 750 mM ε-amino-n-caproic acid) and loaded onto a gel. One-dimensional BN-PAGE and 2D-BN/-Tricine–SDS-PAGE were carried out as described by [24]. If needed, bands of interest were excised from a 1D BN-gel, placed on top of a resolving gel for SDS-PAGE, embedded in stacking gel and subsequently separated by electrophoresis.

### 2.8. Mass spectrometry (MS) analyses

For in-gel digestion stained protein bands and spots were excised, transferred to 0.5 ml siliconised reaction tubes (Ambion, Huntingdon, UK), digested over night with trypsin (Sigma) at 37 °C and extracted as described by [25]. Tryptic peptides were analysed by tandem MS using a quadrupole time-of-flight hybrid mass spectrometer (Q-TOF, Micromass, Altrincham, UK). Conditions for the measurements, instrument settings and description of the procedure are specified in [25]. Partial amino acid sequences were deduced from fragmentation spectra using the PepSeq software (Micromass).

## 3. Results and discussion

### 3.1. Localization of Ycf37

According to a previous study using an antiserum against denatured, recombinant Ycf37 [17], this protein was predominantly found in the thylakoid membrane fraction. To analyze the localization of Ycf37 in more detail, membrane protein complexes solubilized from wild-type thylakoids were separated by sucrose density gradient ultracentrifugation (Fig. 1A) and the obtained fractions immunologically screened by Western blot analysis (Fig. 1B). Three chromophore-containing bands were observed. Polypeptide profiles of all density gradient fractions were examined by SDS-PAGE (data not shown). The upper orange fraction contained, besides others, the abundant red and orange carotenoid proteins [26]. The blue-green band (Fig. 1A; band II) was enriched in PSII monomers but also contained PSI monomers. The dark-green band (Fig. 1A, band III) predominantly consisted of trimeric PSI. All three chromophore containing bands were also present in thylakoids of the Δ*ycf37* mutant, but trimeric PSI complexes only accounted for 70% of the wild-type level (Fig. 1A, band III). The amount of PSI trimers was quantified by determination of the Chl content in the corresponding sucrose gradient fractions, because the Chl-to-P700 ratio was not changed in the mutant [10]. It should be noted that the PSI/PSII monomer fractions (Fig. 1A, band II) of wild type and the *ycf37* mutant had a comparable Chl content.

All fractions of the sucrose gradient were subjected to immunoblot analysis using an Ycf37 antiserum. The immunoblot depicted in Fig. 1B reveals that Ycf37 is mainly located in the orange band in the upper part of the gradient, containing solubilized monomeric proteins but no supramolecular protein complexes. However, significant amounts of Ycf37 were present in the PSI/PSII monomer fraction. The distribution of PSI and PSII complexes was evaluated by simultaneous determination of PsaA and PsaD (subunits of PSI), as well as PsbA and PsbB (subunits of PSII) (Fig. 1B). These data support the view that Ycf37 is either not or only very loosely bound to the photosystems. To estimate whether Ycf37 co-purifies with one of the monomeric PS complexes, the PSI/PSII monomer fraction was purified further by anion exchange chromatography on a Resource Q column. Immunoblot analysis of all fractions showed that the Ycf37 protein was detected only in the flow-through, indicating that the protein was not tightly bound

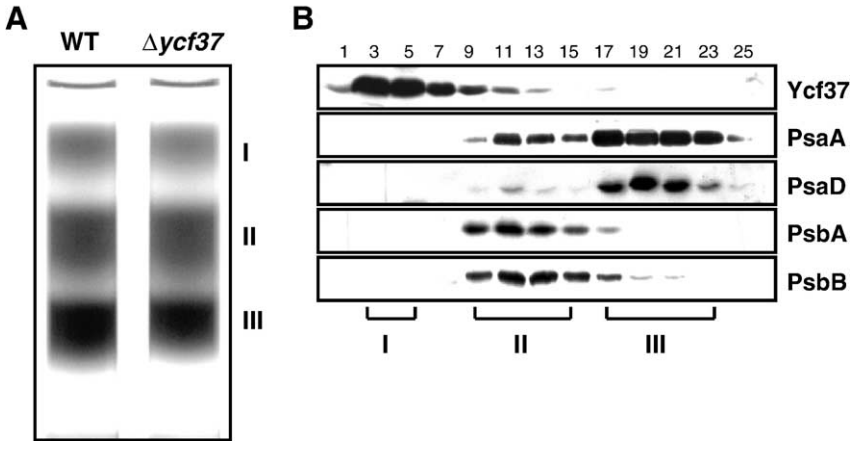


Fig. 1. Subcellular localization of Ycf37. (A) Photographs of detergent solubilized wild-type and Δ*ycf37* mutant thylakoid membranes fractionated by sucrose density gradient ultracentrifugation. Three coloured bands were obtained: (I) carotenoid containing band, (II) PSI/PSII monomers and (III) PSI trimers. Solubilized thylakoids corresponding to 15 mg of protein were applied to each gradient. (B) Fractions of the sucrose gradient from wild-type thylakoids were withdrawn with a syringe, separated by SDS-PAGE and investigated by Western blot analysis using antibodies raised against the Ycf37 protein as well as PSI and PSII subunits. Lane 1 corresponds to the top of the gradient.

to any of the photosystems (data not shown). Although Ycf37 was clearly absent from the soluble cell fraction [17], our results show that the protein can be easily removed from the protein complexes of the thylakoid membrane with the mild detergent β-DM.

Ycf37 contains three TPR-domains. Such a three tandem-TPR motif appears to be the minimal functional unit in TPR-proteins, which typically interact with other proteins or associate to multi-protein complexes [11]. Thus, Ycf37 has the structural prerequisite for an interaction with other target proteins. The relatively loose binding to the thylakoid membrane or its major protein complexes may suggest that Ycf37 interacts transiently with its partners or that solubilization of the membranes with β-DM affects such interactions. In this respect, the Ycf37 protein behaves quite similar to the TPR domain-containing PSI assembling factor Ycf3 [3]. Although Ycf3 interacts with PsaA and PsaD [2], it was found mainly on the top of a sucrose gradient, suggesting a loose association with PSI [3].

### 3.2. Affinity purification of 3xFLAG-tagged Ycf37

In order to specify interaction partners of Ycf37 a system for affinity purification of proteins from *Synechocystis* 6803 was established. Δ*ycf37* mutant cells were engineered to express 3xFLAG-tagged Ycf37 using the self-replicating vector pVZ321. Expression of FLAG-Ycf37 led to a wild-type phenotype of Δ*ycf37* mutant cells suggesting that the tagged protein was fully functional in *Synechocystis* cells (data not shown). Expressed FLAG-Ycf37 was immunoprecipitated with anti-FLAG M2 affinity gel as described in Materials and methods. FLAG-Ycf37 complexes were eluted by the addition of a 3xFLAG peptide and resolved by SDS-PAGE (Fig. 2A). Mutant cells expressing the non-tagged Ycf37 from the pVZ321*ycf37* plasmid were used as a negative control. As shown in Fig. 2A, a prominent band representing FLAG-Ycf37 is present only in the elution fractions from cells that express the tagged protein. Interestingly, these elution fractions contained Chl in contrast to the control samples. In addition, there are multiple bands apparent in SDS-PAGE of the elution fraction but absent in the negative control, suggesting that these bands represent Ycf37-interacting proteins. Two prominent Coomassie-stained bands of ~70 kDa (Fig. 2A) present only in the sample with FLAG-Ycf37 expression were excised from the gel and subjected to trypsin digestion. The resulting tryptic peptides were analysed by ESI tandem MS and partial sequences were deduced from the obtained fragmentation spectra. Based upon these analyses the two 70 kDa bands were found to contain the PsaA and PsaB proteins. To verify that PSI subunits specifically co-purify with FLAG–Ycf37 complexes samples were probed by Western blotting using antibodies directed against PsaA/B, PsaC, PsaD and PsbA (Fig. 2B). As expected, PsaA/B, but also PsaC and PsaD were detected in FLAG–Ycf37 complexes and not in the control sample derived from *Synechocystis* cell expressing only a non-tagged variant of Ycf37. The PSII subunit PsbA was not detected (Fig. 2B), suggesting that PSI subunits specifically interact with Ycf37. However, only a very small proportion of the total cellular pool of PSI seems to co-purify with FLAG-Ycf37. This observation is not surprising as our previous data imply a loose or transient association of Ycf37 with monomeric photosynthetic complexes. Our findings are consistent with the idea that Ycf37 binds only at a specific time point during PSI biogenesis and/or low abundant PSI intermediates. In addition, (an)other protein(s) could be important for Ycf37-PSI interaction that is absent in thylakoid membranes but present in whole cell extracts that were used for FLAG–Ycf37 immunoprecipitation. So, the PSI–Ycf37 interaction would be stabilized in this experimental background in contrast to the anion exchange chromatography experiments (see Section 3.1), where only the thylakoid subfraction was used.

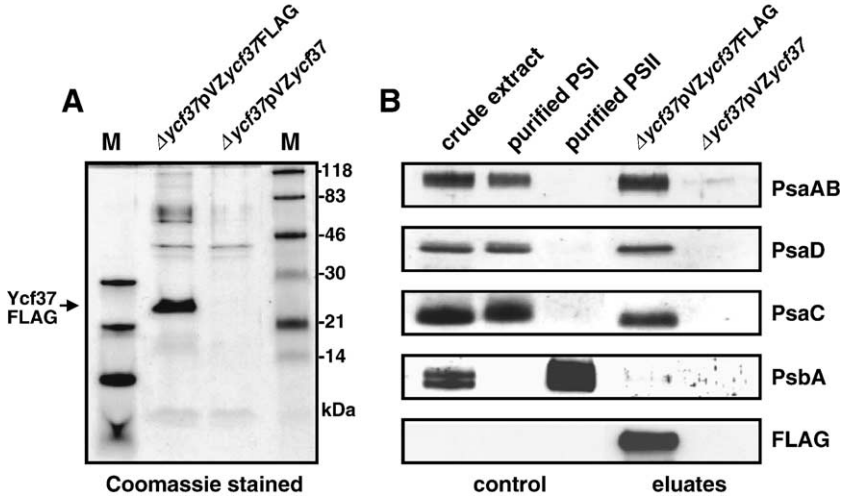


Fig. 2. Affinity purification of 3xFLAG-Ycf37 and identification of PsaA/B, C and D by Western blot analysis. (A) Solubilized extracts of Δ*ycf37* mutant cells engineered to express 3xFLAG–Ycf37 were immunoprecipitated with anti-FLAG M2 agarose. FLAG–Ycf37 complexes were eluted with 3xFLAG peptide, resolved by Tricine–SDS-PAGE and stained with Coomassie blue. Solubilized extracts of Δ*ycf37* mutant cells expressing in trans Ycf37 without a tag served as a control. (B) The presence of PsaA/B, PsaC and PsaD as well as FLAG–Ycf37 in the elution fraction was assessed by Western blotting using specific antibodies. Crude extract as well as purified PSI and PSII fractions were used as control. Antibodies used for the immunoblots are indicated on the right.

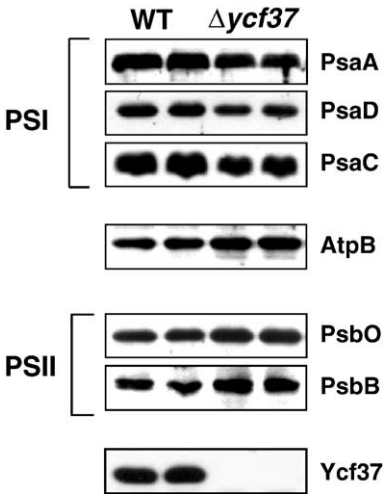


Fig. 3. Semiquantitative Western blot analysis of PSI and PSII subunits in wild-type and Δ*ycf37* mutant thylakoids. Wild-type (WT) and Δ*ycf37* mutant thylakoids equivalent to 25 μg protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Each sample was loaded onto two adjacent lanes. Immunodetection was performed using polyclonal antibodies raised against the PSI subunits PsaA, PsaD, PsaC, and the PSII subunits PsbO and PsbB. An antibody raised against subunit AtpB of the $F_oF_1$–ATP-synthase was used as internal standard. Quantification of the signals revealed a 30–40% reduction of the PSI subunits in the Δ*ycf37* mutant, whereas the amount of PSII subunits was not affected significantly. Antibodies used for the immunoblots are indicated on the right.

### 3.3. Semi-quantitative Western blot analyses of PSI subunits in the wild type and Δycf37 mutant

Previously published low-temperature (77 K) fluorescence emission spectra of intact cells and Western blot analyses of total protein extracts with an anti-PsaA serum indicated a decrease in the PSI/PSII ratio in the Δ*ycf37* mutant [10]. In order to quantify the amounts of individual subunits of the photosystems, specific antisera against various PSI and PSII subunits were used. All signals were normalized to the AtpB signal (Fig. 3). Semi-quantitative analysis (see Section 2.6) of these immunoblots showed that the relative amounts of the PSI subunits PsaA, PsaC and PsaD were reduced in thylakoids of the Δ*ycf37* mutant to about 60–70% of the wild-type level. This result is in line with a 27% reduction of PSI complexes in Δ*ycf37* mutant as judged from spectroscopic measurements ($\Delta A_{703}$) of P700 concentration [10]. By contrast, the amounts of two representative PSII subunits PsbB (CP47) and PsbO were not significantly altered in the mutant (Fig. 3). These data support our presumption that the reduced PSI/PSII ratio of the Δ*ycf37* mutant is caused by a lower PSI content, and not by a PSII accumulation.

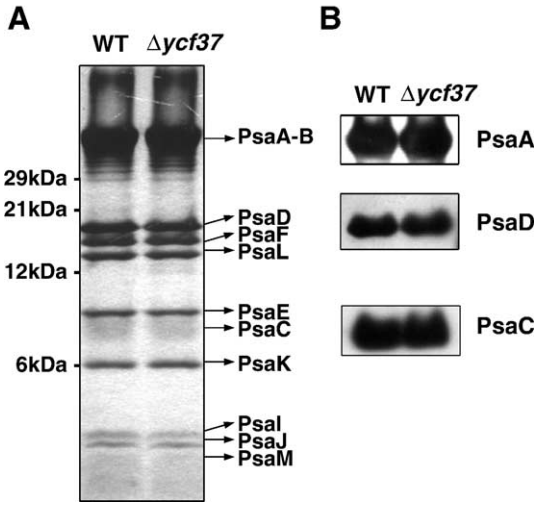


Fig. 4. SDS-PAGE and Western blot analysis of purified PSI-trimers. Ten μg protein of purified wild-type (WT) and Δ*ycf37* mutant PSI complexes after sucrose density gradient centrifugation were separated by Tricine–SDS-PAGE. The proteins were visualized (A) by silver staining or (B) transferred to nitrocellulose membranes. Immunodetection was performed using polyclonal antibodies raised against the PSI subunits PsaA, PsaD and PsaC. Antibodies used for the immunoblots are indicated on the right.

### 3.4. Analysis of trimeric PSI complexes in the Δycf37 mutant

The remaining PSI reaction centres in the Δ*ycf37* mutant were photochemically active, as judged from time-resolved measurements of transient, flash-induced absorbance changes at 703 nm (measured as described in [27]), reflecting the turnover of P700. No significant differences in the number of antenna Chl molecules per P700 were found. In these measurements 144±8.6 Chls per P700 were determined for the wild type and

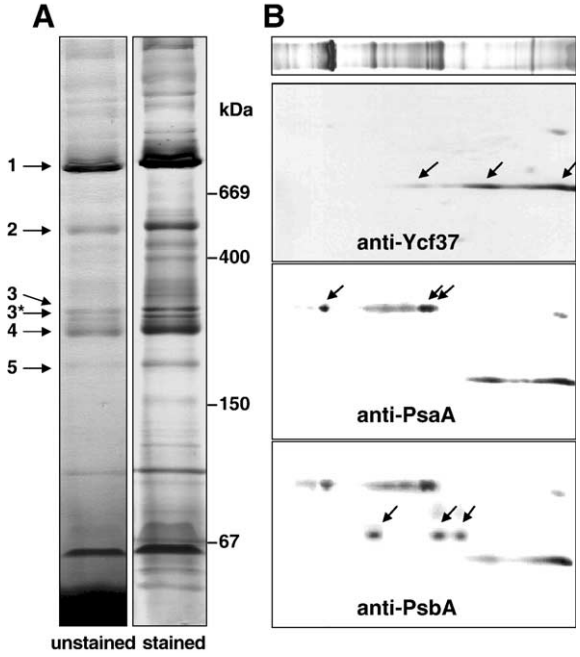


Fig. 5. Membrane protein complexes of *Synechocystis* 6803 wild-type thylakoids separated by BN-PAGE. (A) Unstained and Coomassie-stained BN-gels of wild-type thylakoid membranes. Numbers 1–5 indicate green protein complexes in the unstained gel in accordance to [35] (1, PSI trimer; 2, PSII dimer; 3/3*, PSI monomers; 4, PSII monomer; 5, CP43-less PSII monomer). (B) After separation of the protein complexes in the BN-gel (shown on top of the gel), the lane was excised and subjected to SDS-PAGE (second dimension). The gel from the second dimension was electroblotted to a nitrocellulose membrane and probed sequentially (from top to bottom) with Ycf37, PsaA and PsbA antisera, respectively. Arrows indicate the spots that specifically reacted with the corresponding antiserum.

140±4.2 for the Δ*ycf37* mutant. Furthermore, the P700 re-reduction kinetics were also similar ($\tau_{1/2}$=18 ms±7 ms for the wild type and 17 ms±8 ms for the Δ*ycf37* mutant, respectively).

To test whether the insertional inactivation of the *ycf37* gene had any effect on the subunit composition of the PSI-complex, PSI trimers from the sucrose gradient were analyzed by Tricine–SDS-PAGE and immunoblotting using polyclonal antibodies raised against representative PSI subunits (Fig. 4). All 11 PSI subunits were present in PSI–trimer complexes of the wild type and the Δ*ycf37* mutant (Fig. 4A), with no significant alteration in subunit stoichiometry (Fig. 4B). The fact that in thylakoid membranes of Δ*ycf37* mutant cells reduced levels of PSI subunits accumulate (Fig. 3), and that these PSI complexes are photochemically as active as in the wild type, supports the idea that Ycf37 is involved in PSI biosynthesis and/or stability rather than its function.

Alternatively, the lower PSI content of the *ycf37* mutant cells could be explained by an inhibition of Chl biosynthesis. In cyanobacteria, the biosynthesis of stable PsaA/B proteins depends on supply of Chl [28]. Since most of the Chl is associated with PSI, an inhibition of Chl biosynthesis should likewise lead to a lower PSI content. Indeed, a *chlL* mutant of the cyanobacterium *Plectonema boryanum* being unable to synthesize Chl in the dark, showed a selective decrease in the amount of P700 [29]. He et al. [30] suggested that in cyanobacteria a decreased Chl availability affects the initiation of *psbA* translation. In tobacco plants, Chl deficiency correlated with a drop in the number of PSI as well as PSII centres [31]. However, PSII subunits were not significantly reduced in thylakoid membranes of the *ycf37* mutant (Fig. 3). Furthermore, previous data indicated that light-induced oxygen evolution rates were nearly identical in wild type and mutant when calculated on a per cell basis [10]. Thus, both water cleavage activity and PSII content seem not to be affected in the Δ*ycf37* mutant, supporting the idea of a primary effect of the *ycf37* mutation on PSI assembly and/or stability rather than on Chl biosynthesis. This conclusion is confirmed by the fact that the mutation did not alter the amounts of the Chl precursors protoporphyrin IX and Mg-protoporphyrin IX (data not shown).

### 3.5. Separation of protein complexes using BN/SDS-PAGE

Thylakoid membrane fractions were isolated from wild-type and Δ*ycf37* cells grown under low, intermediate or high light conditions to a cell density corresponding to $OD_{750nm}$=0.8. After solubilization with β-DM, the membranes were subjected to BN-PAGE in order to separate membrane protein complexes.

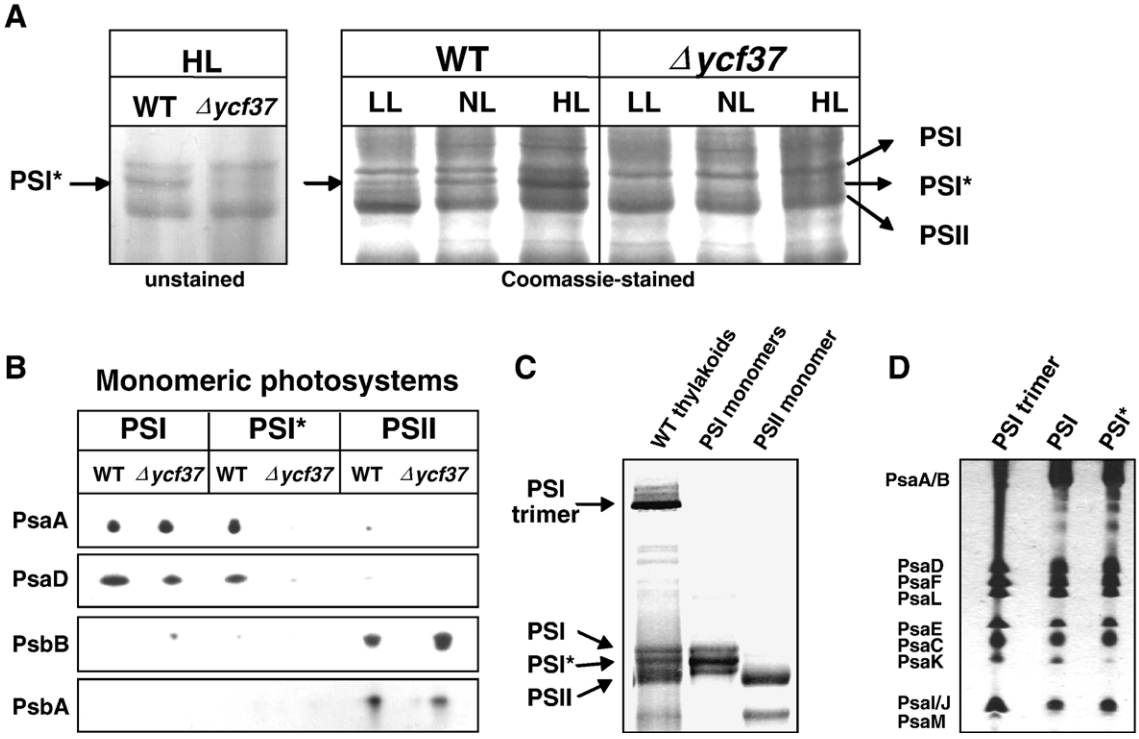


Fig. 6. Analysis of monomeric photosystem complexes of wild-type and Δ*ycf37* mutant cells grown under different light conditions. (A) BN-PAGE of wild-type and Δ*ycf37* mutant thylakoids from cells grown under low (LL, 10 μmol photons $m^{-2}$ $s^{-1}$), medium (NL, 50 μmol photons $m^{-2}$ $s^{-1}$) and high (HL, 170 μmol photons $m^{-2}$ $s^{-1}$) light conditions. Thylakoid membranes were isolated and subjected to BN-PAGE (see Section 2.7). The figure shows unstained and Coomassie-stained sections of the BN-gel including the monomeric photosystems. (B) The bands containing monomeric photosystem complexes (from cells grown under normal light conditions) were excised from the BN-gel, embedded in the stacking gel and subjected to Tricine–SDS-PAGE and transferred to nitrocellulose membranes. PsaA, PsaD, PsbB, and PsbA antibodies were used to reveal the presence of photosystem subunits in the respective complexes. Several blots were used for immunodetection. (C) PSI monomer complexes were purified by anion exchange chromatography and separated on BN-PAGE. As control wild-type thylakoids are shown in lane 1. (D) Bands from the BN-gel containing the purified PSI and PSI* complexes (see panel C, lane 2) were excised, subjected to a second dimension (Tricine–SDS-PAGE) and proteins were visualized by silver staining. A PSI trimer band from separated wild-type thylakoids served as a control (lane 1).

The trimeric PSI complex as well as PSI and PSII monomers were identified by their green colour, originating from Chl associated with the photosynthetic complexes (Fig. 5A). In addition, one-dimensional BN-PAGE was combined with SDS-PAGE and Western blotting to elucidate identities of the resolved complexes on the basis of subunit composition (Fig. 5B). Trimeric PSI was very abundant on BN-gels, whereas monomeric PSI complexes were rather present in small amounts. Quantification of the PSI trimer containing band in BN-gels suggested a 30% decrease of the amount of trimeric PSI in the Δ*ycf37* mutant. This estimate correlates very well with the results obtained by quantifying PSI trimers in sucrose gradients (Fig. 1), by spectroscopic measurements [10] and Western blot analyses (Fig. 3). BN-gel strips containing separated protein complexes of wild-type thylakoids were subjected to SDS-PAGE, and the separated proteins of the second dimension were electrophoretically transferred to nitrocellulose membranes for immunoblot analysis. As shown in Fig. 5B, the Ycf37 protein was spread over the molecular mass range from about 20 to 400 kDa, confirming the broad distribution of Ycf37 in sucrose gradient preparations (Fig. 1B, fractions 1–13). Possibly, Ycf37 is loosely or transiently associated with protein complexes or forms oligomers, as was shown for Ycf3 in vitro [32].

Intermediate and especially high light intensities (50 or 170 μmol photons $m^{-2}$ $s^{-1}$, respectively) during growth promoted the formation of an additional green complex in the wild type, designated PSI*, with a slightly higher electrophoretic mobility in BN-PAGE than the other PSI monomer complex (Fig. 6A). Immunoblots using PSI as well as PSII antibodies (Fig. 6B) clearly indicated the distinct presence of PSI subunits in the PSI* complex. PSI* was not found in the Δ*ycf37* mutant, even under high light conditions (Fig. 6A). At this point it should be noted that under high light conditions the growth rate of the Δ*ycf37* mutant is significantly reduced to about 80% of the wild type, whereas no growth retardation was detected under low light conditions (10 μmol photons $m^{-2}$ $s^{-1}$).

To verify this phenotype, the Δ*ycf37* mutation was complemented by introduction of the conjugative vector pVZ*ycf37*, which carries the wild-type *ycf37* gene including its promoter, into Δ*ycf37* mutant cells. Exconjugants harbouring this plasmid had wild-type Chl and PSI contents but an enhanced amount of Ycf37 (data not shown). Because the introduced *ycf37* gene was under control of its own promoter, the higher Ycf37 protein level was possibly due to the number of pVZ*ycf37* plasmid copies in the cells. Thylakoid membrane preparations from complemented mutant cells again exhibited the PSI* complex that was absent from the original Δ*ycf37* mutant (Fig. 7). These results clearly establish that the absence of PSI* in Δ*ycf37* mutant thylakoids is a consequence of the Δ*ycf37* mutation and rule out any defect caused by a secondary mutation.

In order to investigate the nature of PSI*, PSI monomer complexes were purified by anion exchange chromatography and separated by BN-PAGE (Fig. 6C, lane 2). Bands containing PSI and PSI* were excised and subjected to a denaturating second dimension (Fig. 6D). No difference in the subunit composition between the PSI trimers (Fig. 6D, lane 1) and the PSI monomer (Fig. 6D, lane 2) was detected. However, in PSI* complexes, the amount of PsaK seems to be reduced significantly (Fig. 6D, lane 3) in relation to the other PSI subunits. The low abundant PsaK subunit in PSI* could be also a contamination by the other PSI monomer complex because of the close neighbourhood of these two bands in the BN-gel (Fig. 6C). Thus, we suggest that one possible explanation for the higher mobility of PSI* is the absence of PsaK. In Tricine–SDS-PAGE, the three small PSI subunits PsaI, J, and M at about 3–4 kDa were evenly evident in both PSI monomer complexes but we were not able to exactly assign them (Fig. 6D). In addition, we cannot exclude that a different cofactor composition or posttranslational modification contribute also to the different mobility of both PSI monomer complexes. Fig. 6C shows an additional third PSI monomer complex that appears after anion exchange chromatography. This complex lacks PsaL and PsaK (data not shown) and is possibly a decomposition product of the purification procedure. It should be noted that Ycf37 is obviously not associated with PSI monomer preparations after anion exchange chromatography on a Resource Q column (see Section 3.1). Thus, we can exclude that binding of Ycf37 is responsible for the different mobility of the PSI monomer complexes.

In summary, our results show that Ycf37 can be easily removed from the protein complexes of the thylakoids by the

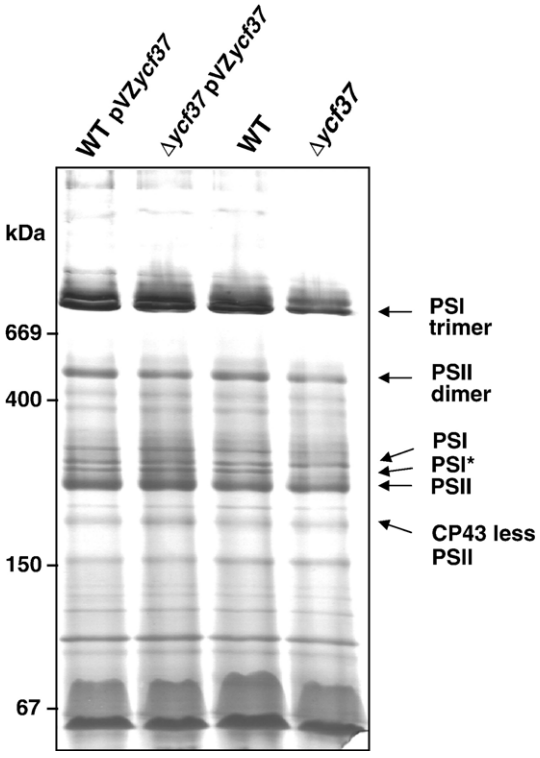


Fig. 7. Complementation of the Δ*ycf37* mutant phenotype. The Δ*ycf37* mutant strain was complemented by the conjugative vector pVZ321 expressing an *ycf37* wild-type gene copy under control of its own promoter (pVZ*ycf37*). Protein complexes from solubilized thylakoids of wild type (WT), Δ*ycf37*, WTpVZ*ycf37* and Δ*ycf37*pVZ*ycf37* strains were separated by BN-PAGE and stained with Coomassie.

mild detergent β-DM. However, a small amount of Ycf37 was detected in the PSI/PSII monomer fraction, suggesting a transient association with one or both of these complexes. This conclusion is also supported by co-purification of individual PSI subunits with a 3xFLAG-tagged Ycf37 protein from *Synechocystis* cells. Mutation of *ycf37* causes a reduction of the PSI trimer content by approx. 30%. The organization of the remaining PSI trimers in the *ycf37* mutants was indistinguishable from the wild-type complexes, which is in line with the observation that PSI is fully active in this mutant. Therefore, Ycf37 alone is not essential for correct assembly of cyanobacterial PSI. Although Ycf37 might be substituted in part by another unknown assembling factor, it may either stabilize the monomeric PSI reaction centre or promote its biogenesis. However, the data presented in this work did not reveal a definite interaction of Ycf37 with PSI*. The lower amount of PSI trimers in the *ycf37* mutant could indicate that the PSI* complex is an intermediate in a dynamic assembly/disassembly cycle of trimeric complexes. The number of PSI complexes decreases in vivo at high light intensity [33]. Since PSI exists mainly as a trimer in cyanobacteria, high light intensities lead to a destabilization of PSI trimers and to an accumulation of an intermediate form, designated PSI*. We therefore suggest that PSI* potentially is a pool of monomeric PSI that can be recycled to form PSI trimers if needed. This pool might be unstable in Δ*ycf37* mutant cells leading to a lower content of PSI trimers. The alternative PSI subunit PsaK2 is incorporated into the PSI complexes during high light acclimation [34]. The lower abundance or absence of PsaK in PSI* (we were not able to differentiate between PsaK1 and PsaK2 in our experiments) may perhaps reflect a function of Ycf37 in the stabilization of intermediate PSI complexes that acclimate to high light changing PsaK1 to PsaK2. This could lead to a disadvantage in growth of the Δ*ycf37* mutant in comparison to the wild type under high light conditions.

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## References

[1] T. Cavalier-Smith, Membrane heredity and early chloroplast evolution, Trends Plant Sci. 5 (2000) 174–182.
[2] H. Naver, E. Boudreau, J.D. Rochaix, Functional studies of Ycf3: its role in assembly of photosystem I and interactions with some of its subunits, Plant Cell 13 (2001) 2731–2745.
[3] E. Boudreau, Y. Takahashi, C. Lemieux, M. Turmel, J.-D. Rochaix, The chloroplast *ycf3* and *ycf4* open reading frames of *Chlamydomonas reinhardtii* are required for accumulation of photosystem I complex, EMBO J. 16 (1997) 6095–6104.
[4] S. Ruf, H. Kössel, R. Bock, Targeted inactivation of a tobacco intron-containing open reading frame reveals a novel chloroplast-encoded photosystem I-related gene, J. Cell Biol. 139 (1997) 95–102.
[5] Z. Xie, S. Merchant, The plastid-encoded *ccsA* gene is required for heme attachment to chloroplast c-type cytochromes, J. Biol. Chem. 271 (1996) 4632–4639.
[6] Y. Takahashi, M. Rahire, C. Breyton, J.L. Popot, P. Joliot, J.D. Rochaix, The chloroplast *ycf7* (*petL*) open reading frame of *Chlamydomonas reinhardtii* encodes a small functionally important subunit of the cytochrome $b_6f$ complex, EMBO J. 15 (1996) 3498–3506.
[7] M. Swiatek, R. Kuras, A. Sokolenko, D. Higgs, J. Olive, G. Cinque, B. Müller, L.A. Eichacker, D.B. Stern, R. Bassi, R.G. Herrmann, F.A. Wollman, The chloroplast gene *ycf9* encodes a photosystem II (PSII) core subunit, PsbZ, that participates in PSII supramolecular architecture, Plant Cell 13 (2001) 1347–1367.
[8] M.E. Reith, J. Munholland, Complete nucleotide sequence of the *Porphyra purpurea* chloroplast genome, Plant Mol. Biol. Rep. 13 (1995) 333–335.
[9] S.E. Douglas, S.L. Penny, The plastid genome of the cryptophyte algae *Guillardia theta*: complete sequence and conserved synteny groups confirm its common ancestry with red algae, J. Mol. Evol. 48 (1999) 236–244.
[10] A. Wilde, K. Lünser, F. Ossenbühl, J. Nickelsen, T. Börner, Characterization of the cyanobacterial *ycf37*: mutation decreases the photosystem I content, Biochem. J. 357 (2001) 211–216.
[11] L.D. D'Andrea, L. Regan, TPR proteins: the versatile helix, Trends Biochem. Sci. 28 (2003) 655–662.
[12] T. Schwabe, J. Kruip, Biogenesis and assembly of photosystem I, Indian J. Biochem. Biol. 37 (2000) 351–359.
[13] J.D. Rochaix, K. Perron, D. Dauvillee, F. Laroche, Y. Takahashi, M. Goldschmidt-Clermont, Post-transcriptional steps involved in the assembly of photosystem I in *Chlamydomonas*, Biochem. Soc. Trans. 32 (2004) 567–570.
[14] K. Amann, L. Lezhneva, G. Wanner, R.G. Herrmann, J. Meurer, ACCUMULATION OF PHOTOSYSTEM ONE1, a member of a novel gene family, is required for accumulation of [4Fe–4S] cluster-containing chloroplast complexes and antenna proteins, Plant Cell 16 (2004) 3084–3097.
[15] T. Yabe, K. Morimoto, S. Kikuchi, K. Nishio, I. Terashima, M. Nakai, The *Arabidopsis* chloroplastic NifU-like protein CnfU, which can act as an iron–sulfur cluster scaffold protein, is required for biogenesis of ferredoxin and photosystem I, Plant Cell 16 (2004) 993–1007.
[16] G. Shen, J. Zhao, S.K. Reimer, M.L. Antonkine, Q. Cai, S.M. Weiland, J. H. Golbeck, D.A. Bryant, Assembly of photosystem I: I. Inactivation of the *rubA* gene encoding a membrane-associated rubredoxin in the cyanobacterium *Synechococcus* sp. PCC 7002 causes a loss of photosystem I activity, J. Biol. Chem. 277 (2002) 20343–20354.
[17] U. Dühring, A. Karradt, S. Mikolajczyk, K.-D. Irrgang, A. Wilde, Functional investigations on conserved chloroplast ORFs from the cyanobacterium *Synechocystis* sp. PCC 6803, in: A. van der Est, D. Bruce (Eds.), Photosynthesis: Fundamental Aspects to Global Perspectives: Proceedings 13 th International Congress on Photosynthesis, Alliance Communications Group Publ., Lawrence, Montreal, Canada, 2005, pp. 802–804.
[18] R. Rippka, J. Deruelles, J.B. Waterbury, M. Herdmann, R.T. Stanier, Genetic assignment, strain histories and properties of pure cultures of cyanobacteria, J. Gen. Microbiol. 111 (1979) 1–61.
[19] V.V. Zinchenko, I. Piven, V. Melnik, S. Shestakov, Vectors for complementation analysis of mutants of cyanobacteria, Genetika 35 (1999) 291–296.
[20] M. Rögner, P.J. Nixon, B.A. Diner, Purification and characterization of photosystem I and photosystem II core complexes from wild-type and phycocyanin-deficient strains of the cyanobacterium *Synechocystis* PCC 6803, J. Biol. Chem. 265 (1990) 6189–6196.
[21] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[22] H. Schägger, G. von Jagow, Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for separation of proteins in the range of 1 to 100 kDa, Anal. Biochem. 166 (1987) 368–379.

[23] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[24] L. Jänsch, V. Kruft, U.K. Schmitz, H.P. Braun, Cytochrome c reductase from potato does not comprise three core proteins but contains an additional low-molecular-mass subunit, Eur. J. Biochem. 228 (1995) 878–885.
[25] C. Walz, M. Juenger, M. Schad, J. Kehr, Evidence for the presence and activity of a complete antioxidant defence system in mature sieve tubes, Plant J. 31 (2002) 189–197.
[26] Y.P. Wu, D.W. Krogmann, The orange carotenoid protein of *Synechocystis* sp, PCC 6803, Biochim. Biophys. Acta 1322 (1997) 1–7.
[27] J. Miao, A. Wilde, R. Jordan, E. Schlodder, T. Börner, K.-D. Irrgang, Structural and functional investigations on the Ycf3, Ycf4 and Ycf37 proteins in the cyanobacterium *Synechocystis* sp. PCC 6803, Proceedings of the 12th International Congress on Photosynthesis, Brisbane, Australia, CSIRO Publishing, Collingwood, Australia, 2001, pp. S23–S007.
[28] K. Aizawa, Y. Fujita, Regulation of synthesis of PSI in the cyanophytes *Synechocystis* PCC 6714 and *Plectonema boryanum* during the acclimation of the photosystem stoichiometry to the light quality, Plant Cell Physiol. 38 (1997) 319–326.
[29] S. Kada, H. Koike, K. Satoh, T. Hase, Y. Fujita, Arrest of chlorophyll synthesis and differential decrease of photosystems I and II in a cyanobacterial mutant lacking light-independent protochlorophyllide reductase, Plant Mol. Biol. 51 (2003) 225–235.
[30] Q. He, D. Brune, R. Nieman, W. Vermaas, Chlorophyll a synthesis upon interruption and deletion of por coding for the light-dependent NADPH: protochlorophyllide oxidoreductase in a photosystem-I-less/*chlL*-strain of *Synechocystis* sp, PCC 6803, Eur. J. Biochem. 253 (1998) 161–172.
[31] H. Härtel, E. Kruse, B. Grimm, Restriction of chlorophyll synthesis due to expression of glutamate 1-semialdehyde aminotransferase antisense RNA does not reduce the light-harvesting antenna size in tobacco, Plant Physiol. 113 (1997) 1113–1124.
[32] T.M.E. Schwabe, K. Gloddek, D. Schluesener, J. Kruip, Purification of recombinant BtpA and Ycf3, proteins involved in membrane protein biogenesis in *Synechocystis* PCC 6803, J. Chromatogr. 786 (2003) 45–59.
[33] Y. Fujita, A. Murakami, K. Aizawa, Short-term and long-term adaptation of the photosynthetic apparatus: homeostatic properties of thylakoids, in: D.A. Bryant (Ed.), The Molecular Biology of Cyanobacteria, Kluwer Academic Publishers, Dordrecht, 1994, pp. 677–692.
[34] T. Fujimori, Y. Hihara, K. Sonoike, PsaK2 subunit in photosystem I is involved in state transition under high light condition in the cyanobacterium *Synechocystis* sp. PCC 6803, J. Biol. Chem. 280 (2005) 22191–22197.
[35] M. Herranen, N. Battchikova, P. Zhang, A. Graf, S. Sirpiö, V. Paakkarinen, E.-M. Aro, Towards functional proteomics of membrane protein complexes in *Synechocystis* sp. PCC 6803, Plant Physiol. 134 (2004) 470–481.