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Differential requirement of two homologous proteins encoded by *sll1214* and *sll1874* for the reaction of Mg protoporphyrin monomethylester oxidative cyclase under aerobic and micro-oxic growth conditions

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ABSTRACT

The two open reading frames in the *Synechocystis* sp. PCC 6803 genome, *sll1214* and *sll1874*, here designated *cycI* and *cycII*, respectively, encode similar proteins, which are involved in the Mg protoporphyrin monomethylester (MgProtoME) cyclase reaction. The impairment of tetrapyrrole biosynthesis was examined by separate inactivation of both cyclase encoding genes followed by analysis of chlorophyll contents, MgProtoME levels and several enzyme activities of tetrapyrrole biosynthesis. We additionally addressed the question, whether the two isoforms can complement cyclase deficiency under normal aerobic and micro-oxic growth conditions in light. A *cycII* knock-out mutant grew without any adverse symptoms at normal air conditions, but showed MgProtoME accumulation at growth under low oxygen conditions. A complete deletion of *cycI* failed in spite of mixotrophic growth and low light at both ambient and low oxygen, but resulted in accumulation of 150 and 28 times more MgProtoME, respectively, and circa 60% of the wild-type chlorophyll content. The CycI deficiency induced a feedback-controlled limitation of the metabolic flow in the tetrapyrrole biosynthetic pathway by reduced ALA synthesis and Fe chelatase activity. Ectopic expression of the CycI protein restored the wild-type phenotype in *cycI*$^-$ mutant cells under ambient air as well as micro-oxic growth conditions. Overexpressed CycII protein could not compensate for *cycI*$^-$ mutation under micro-oxic and aerobic growth conditions, but complemented the *cycII* knock-out mutant as indicated by wild-type MgProtoME and chlorophyll levels. Our findings indicate the essential contribution of CycI to the cyclase reaction at ambient and low oxygen conditions, while low oxygen conditions additionally require CycII for the cyclase activity.

1. Introduction

The various end products of the tetrapyrrole biosynthetic pathway, protoheme, siroheme, phycobilines and chlorophyll, serve in photosynthetic organisms as essential cofactors and prosthetic groups in many biochemical processes [1–6]. (Bacterio-) chlorophylls and the group of phycobilines are essential for photosynthetic light absorbance. Pigments and the pigment-binding proteins are present in stoichiometric amounts predicting a control mechanism that coordinates the synthesis of apoproteins and their associated pigments [7].

Consequently, the photosynthetic capacity is closely connected to tetrapyrrole biosynthesis. Most of the more than fifteen enzymatic steps leading from glutamyl-tRNA to chlorophyll are well understood and biochemically and genetically characterized. Analysis of mutants and transgenic photosynthetic bacteria, algae and plants as well as of recombinant proteins of tetrapyrrole biosynthesis generated a huge – and still growing – body of information about the control of expression and biochemical activities of this metabolic pathway [1–6]. It is assumed that the dynamic variation in the demand of all tetrapyrrole end products requires a tight control of the metabolic flow within the branched pathway.

More recently, new findings on metabolic control and plastid-to-nucleus signalling made the chlorophyll-synthesizing Mg branch of the tetrapyrrole pathway to an attractive research area [1,4,8]. Among the enzymatic steps of the Mg branch, the complex catalytic conversion of Mg protoporphyrin IX monomethylester (MgProtoME) to protochlorophyllide resulting in the formation of the isocyclic ring E of Mg porphyrins was still less studied. Apart from the metabolic substrate MgProtoME this reaction requires NADPH, iron and oxygen. Oxygen can be incorporated in this catalytic reaction by two different mechanisms, which are used by an oxygen-independent or a -dependent cyclase.

Abbreviations: ALA, 5-aminolevulinic acid; MgProto, Mg protoporphyrin IX; MgProtoME, Mg protoporphyrin IX monomethylester; Proto, protoporphyrin IX

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During anaerobiosis the introduced oxygen atom at the C13[1] position originates from water catalyzed by a hydratase [9], while under aerobic conditions the same 13[1]-oxogroup is derived from molecular oxygen introduced by an oxygenase [10]. Oxygen is introduced by a stereospecific hydroxylation at the β-position of the propionate side chain of ring C followed by oxidation of the hydroxyl group to a carbonyl group and oxidative ring formation at the C-α position leading to the cyclopentanon ring E [11].

Mutant gene analysis of the photosynthetic operon of *Rhodobacter* species performing anaerobic photosynthesis revealed accumulation of MgProtoME upon interruption of the *bchE* gene suggesting the encoded protein to be involved in the oxygen-independent cyclase reaction [12]. The purple bacterium *Rubrivivax gelatinosus* synthesizes bacteriochlorophyll *a* under both aerobic and anaerobic conditions [13,14]. An *acsF* (aerobic cyclisation) mutant accumulates MgProtoME and revealed with AcsF a first gene product, which is involved in the oxygen-dependent cyclase reaction of MgProtoME (as from now the enzyme is abbreviated by cyclase) [13]. The deletion of the *bchE* gene impaired bacteriochlorophyll synthesis of *Rubrivivax* under low oxygen concentration and in anaerobic conditions [14].

Genes with sequences similar to *acsF* were found in prokaryotic and eukaryotic organisms possessing oxygenic photosynthesis [8,15–17]. The first descriptions of these similar genes in plants and the green algae *Chlamydomonas reinhardtii* did not directly assign the encoded proteins to the metabolic pathway of tetrapyrrole biosynthesis. A homologous gene *PNZIP* of the plant *Pharbitis nil* was initially described to be light induced and circadian clock controlled. The corresponding protein contained a leucine zipper motif, which is apparently important for protein–protein interaction [15]. A mutant screen under copper deficiency led to the identification of the *Chlamydomonas crd1* gene (*copper response defect 1*). Its deficiency led to reduced photosystem I assembly [8]. Furthermore, a second homologous gene *cth1* was found in the *Chlamydomonas* genome [18]. *Crd1* is expressed under low aeration and/or copper starvation, whereas *cth1* is expressed under oxygenated and copper-sufficient conditions. More recently, δ-aminolevulinic acid (ALA) feeding to the *Chl27* antisense expressing *Arabidopsis thaliana* seedlings resulted in MgProtoME accumulation and assigned this *acsF* homolog to the cyclase reaction [18,19].

The aerobic and anaerobic cyclase proteins have no structural similarity. While the oxygen-dependent AcsF protein is characterized by a metal-binding site consisting of the two (D/E)X_nEXXH motives belonging to the family of diiron carboxylate proteins [20], the BchE protein possesses a CXXXCXXC domain which is conserved in different proteins involved in anaerobic processes.

In cyanobacterial genomes one or two genes encoding AcsF-homologous proteins were found with the exception of the *Nostoc* sp. genome (PCC 7120) having three copies with a similar gene sequence (*alr3300*, *alr1358*, *alr1880*). These gene sequences were assigned to a group of genes, which were previously described as *ycfs* (hypothetical chloroplast open reading frames) [21]. *Ycfs* were initially found to be conserved in the chloroplast genomes of plants and algae. Sometimes they have been transferred to the nuclear genome during evolution. Most of these *ycfs* are also found in the cyanobacterial genome confirming the contribution of *ycfs* to common functions in photosynthetic organisms.

The *Synechocystis* sp. PCC 6803 genome contains two genes with similarity to *acsF*, *sll1214* and *sll1874* annotated as *ycf59* in the Cyanobase data bank. Minamizaki et al. [17] knocked out both *acsF* homologous genes designated *chlAI* and *chlAII* and three *bchE*-like genes in *Synechocystis* sp. PCC 6803. Interestingly, *sll1874* is apparently required for growth under low oxygen conditions, while the protein encoded by *sll1214* is essential for normal aerobic growth conditions. Open questions remained about the regulatory consequences of reduced cyclase activity on the pathway of tetrapyrrole biosynthesis and the role and requirement of both cyclase proteins under normal aerobic and micro-oxic conditions. We generated *Synechocystis* mutants for both cyclase genes to explore the consequences of compromised cyclisation reaction for the metabolic flow of chlorophyll intermediates and enzyme activities in the tetrapyrrole biosynthetic pathway. Moreover, we examined, whether the two *Synechocystis ycf59* genes can complement cyclase deficiency in ambient and low oxygen conditions.

2. Materials and methods

2.1. Culture conditions

Synechocystis sp. PCC 6803 wild type – the strain was originated from the laboratory of S. Shestakov, Moscow State University – and mutant strains were grown at 30 °C in a BG-11 medium [22] containing 0.2% glucose under continuous irradiance of 20 μmol photons m^{-2} s^{-1} in rotating Erlenmeyer flasks for ambient air conditions. For micro-oxic conditions aerobically precultured cells were further bubbled with N_2 in a fermenter for 2–3 days. Control cells were grown under the same conditions and bubbled with ambient air.

2.2. Mutagenesis

The *ycf59* loci *sll1214* (*cycI*) and *sll1874* (*cycII*) were amplified using primers P1 (5′-CCGTTTGCAACAAAACACGTAA-3′) and P2 (5′-ACAGCGGGATTAATCAGCCTTC-3′) or P3 (5′-TTAGGCTAAATCAGGGGC AACA-3′) and P4 (5′-CCAAAGGTAATTAGCAATAATCAGCA-3′). The amplicons contained the flanking regions of *sll1214* and *sll1874*, yielding PCR products of 1558 bp or 1677 bp, respectively. These products were subcloned into pGEM-T (Promega, Mannheim, Germany). The kanamycin resistance cassette from pUC4K (New England Biolabs, Frankfurt/Main, Germany) was introduced into a single SmaI site to disrupt the *sll1214* sequence or between two BsaBI sites in ORF *sll1874* (Fig. 2). Transformants were selected on solid BG-11 media with increasing concentrations of kanamycin (up to 120 μg ml^{-1}) and analysed by Southern blot hybridization and PCR using primers 5′-CTTCCGCTGGATCACCTTTA-3′ and 5′-GGTAATGACTTCCCCGAAGA-3′ for *sll1214* or 5′-AAATTGAAGCGGCGTTGG-3′ and 5′-CTAGCCCCACCGA CTGATAA-3′ for *sll1874* to detect the level of segregation of mutant gene copies.

2.3. Determination of porphyrin steady-state levels

Steady-state levels of porphyrins and Mg porphyrins were analysed from cell cultures grown in the logarithmic growth phase. Ten ml of cultures was centrifuged and the cell pellets were subsequently frozen at −80 °C. Porphyrins were extracted by resuspending each pellet in a 250 μl porphyrin extraction buffer (acetone/methanol/0.1 M NH_4OH (10/9/1; v/v/v)) and incubated on ice for 30 min. After 15 min centrifugation the supernatant was transferred to a new tube and extraction was repeated twice. The supernatants were combined for HPLC analysis. Extracted porphyrinogens were oxidized by addition of 5 μl 1 M acetic acid and 5 μl 2-butanone peroxide per 200 μl extract. Porphyrins were analysed by HPLC (Agilent-1100, Agilent, Waldborn, Germany) using an RP 18 column at a flow rate of 1 ml min^{-1} (Novapak C18, 4 μm particle size, 3.9×150 mm; Waters Chromatography, Eschborn, Germany). Porphyrins were eluted with a linear gradient of solvent A (10% methanol, 0.1 M ammonium acetate, pH 5.2) and solvent B (90% methanol, 0.1 M ammonium acetate, pH 5.2) as follows: 0–100% over 7 min followed by 100% solvent B for 17 min. The eluate was monitored by fluorescence detection. Excitation and emission wavelengths for protoporphyrin IX (Proto) and Mg protoporphyrin IX (MgProto)/MgProtoME were 405 nm and 625 nm or 420 nm and 595 nm, respectively. Porphyrins and Mg porphyrins were identified and quantified by using authentic standards (Frontier Scientific, Logan, USA).

2.4. Absorption spectra and pigment measurement

Absorption spectra of whole cells were measured with a UV-2401 PC spectrophotometer (Shimadzu). Chlorophyll contents were measured of whole cell lysates in 90% methanol according to [23]. Phycocyanin contents were determined in the soluble protein fraction of cell extracts [24].

2.5. Enzyme assays

For measuring Mg chelatase activity *Synechocystis* cell cultures (800 ml) were pelleted, disrupted with glass beads in cold isolation buffer (50 mM Tricine, pH 7.8, 0.5 M sorbitol, 5 mM $MgCl_2$ and 1 mM DTT) and the protein concentration of the homogenate was adjusted to 2 mg ml^{-1}. Fifty μl of the pre-warmed homogenate was mixed with 50 μl 2× assay mix (3 μM Proto, 10 mM ATP, 10 mM Tricine, pH 7.8). The reaction was stopped after 0, 10, 20 and 30 min by adding 400 μl ice-cold acetone. Hexane was used to remove chlorophyll from the extract. Finally, Mg porphyrins formed during incubation were measured spectrofluorimetrically (Hitachi F4500 fluorescence spectrophotometer) and amounts were calculated using recorded calibration curves with MgProto (Frontier Scientific, Logan, USA).

For determination of ferrochelatase activity cells of 50 ml liquid cultures were centrifuged, re-suspended in buffer containing 20 mM HEPES, pH 7.6, 1 mM DTT, 10 mM $MgCl_2$, 5 mM $CaCl_2$ and 0.5 M mannitol and disrupted with glass beads before the resulting homogenates were solubilized by adding β-dodecylmaltosid to a final concentration of 0.5% and finally centrifuged at 32,000 *g* for 10 min to remove unbroken cells. Ferrochelatase activity was monitored spectrofluorometrically at 34 °C by on-line recording of the rate of Zn protoporphyrin formation (Hitachi F4500 fluorescence spectrophotometer). The reaction mixture contained 0.3 M TRIS, pH 8.0, 0.03% Tween 80, 2 μM Proto and 2 μM $ZnSO_4$. The reaction was started by adding 20 μg protein from the prepared homogenate to reach the final reaction volume of 1.5 ml. The amount of the catalytic product formed during incubation was calculated by recording calibration curves with Zn protoporphyrin (Frontier Scientific, Logan, USA).

ALA synthesizing capacity was determined from *Synechocystis* cells which were incubated in a BG-11 medium containing 40 mM levulinic acid (pH 7.2) for 4 h under normal light conditions. Cells were centrifuged, disrupted in 1 ml 20 mM K-phosphate buffer, pH 6.8, and unbroken cells were removed by centrifugation. 400 μl of the supernatant was mixed with 100 μl ethylacetoacetate, boiled for 10 min and subsequently chilled on ice before one volume of Ehrlich's reagent was added and ALA derivatives were quantified at $\lambda = 553$ nm.

2.6. RNA procedures

RNA was extracted from *Synechocystis* cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. After electrophoresis RNA was blotted onto a Nylon membrane and hybridized with a *sll1214* fragment amplified by PCR. For semi-quantitative PCR 1 μg of extracted RNA was converted into cDNA by using RevertAid™ M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's advice. The cDNA was diluted and equal amounts were used for RT-PCR using primers 5′-ACTGAGGGACGAAAGCTAGGG-3′ and 5′-GTGTGTAGCCCAAGGCGTAAG-3′ (for amplification of 16S rRNA) and primer pairs 5′-ATCCCAACGATTGCATTTATCC-3′ and 5′-TGCAATGGGC TTCATCAAATAC-3′ as well as 5′-ACTTTCTTCCCGCCAGAGTG-3′ and 5′-GCGATCGCCGTTAGTTTTTC-3′ for quantification of transcripts from *sll1214* and *sll1874*, respectively.

2.7. Western blots

Proteins were separated by SDS-PAGE [25], transferred onto nitrocellulose membranes and immunolabeled with specific antibodies. Signals were visualized using the Immobilon Western membranes chemiluminescence detection system (Millipore, Bedford, USA) or chromogenic detection (alkaline phosphatase with 5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt and nitro-blue tetrazolium chloride), respectively. Anti-ChlI, anti-ChlH, anti-ChlI and anti-ChlD sera were kindly provided by Prof. C.N. Hunter, University of Sheffield, UK; anti-AtpB by Dr. K.-D. Irrgang, Tech. University, Berlin. Anti-HemH was generated for our laboratories and anti-CycI purchased from Agrisera, Sweden

2.8. Construction of inducible gene constructs encoding 3×FLAG-tagged CycI and CycII proteins

The coding sequences of the *Synechocystis* sp. PCC 6803 *cycI* and *cycII* genes were amplified using the primers 5′-AACCATATGATGGTTA ATACCCTCGAAAAGCC-3′ and 5′-ACAAGATCTTTAGCGCACAGCTC CAGC-3′ for *cycI* and 5′-GAACATATGATGGTATCCACTACCCTAC CGACT-3′ and 5′-CAAAGATCTTTAACACACCATCCCCCG-3′ for *cycII*. An NdeI site that overlaps with the ATG start codon of the respective gene was introduced by the forward primer. The PCR product was subcloned into the pDrive vector (Qiagen, Hilden, Germany). Two complementary oligonucleotides encoding the 3×FLAG peptide (according to Sigma-Aldrich, Steinheim, Germany) were hybridized, thus creating NdeI compatible overhangs, and then ligated into the respective site of the pDrive-cyclase constructs. The correctness of the resulting constructs was confirmed by sequencing. Using NdeI and BglII restriction enzymes DNA fragments encoding the FLAG-tagged cyclase fusion proteins were excised and then ligated into pSK9 vector (S. Zinchenko, unpublished) under control of the copper dependent *petJ* promoter [26]. *cycI* deficient cells were transformed using these suicide vectors (*pSKCycI-FLAG* and *pSKCycII-FLAG*). Transformants were selected on BG-11 agar plates containing 7 μg ml^{-1} chloramphenicol. For overexpression of the fusion protein under control of the *petJ* promoter cells were grown for at least 7 days in a BG-11 medium lacking copper in the trace metal mix.

3. Results

The two open reading frames *sll1214* and *sll1874* encode each a protein with 358 amino acid residues and a molecular weight of 42.2 kDa and 41.9 kDa, respectively. Instead of *chlAI* and *chlAII* [17], we introduce the abbreviation *cycI* and *cycII* for the two homologous genes, as this designation defines more precisely the potential function of the encoded protein in chlorophyll biosynthesis. Using the Blast P algorithm the protein sequence comparison indicates that the two proteins CycI and CycII contain 57% identical amino acid residues. The similarity of the amino acid sequence between the *Arabidopsis thaliana* homolog CHL27 and CycI and CycII is 58% and 47%, respectively. All similar proteins from prokaryotes and eukaryotes found in the sequence data bank derived from photosynthetic organisms. Both *Synechocystis* sp. PCC 6803 *cyc* genes are placed in potential operons close to other open reading frames involved in tetrapyrrole biosynthesis: *cycI* (*sll1214*) is located on the *Synechocystis* genome next to *ycf53* (*sll0558*), which encodes the plant homolog GUN4 protein [27], while *sll1874* is positioned in close proximity to *sll1875* (*ho2*) and *sll1876* (*hemN1*) coding for one of the two heme oxygenase isoforms and the oxygen-independent coproporphyrinogen III oxidase, respectively.

3.1. The phenotype of the Synechocystis PCC 6803 mutants cycI^{-} and ΔcycII and cycII

The two *cyc* genes were inactivated in the PCC strain of *Synechocystis* 6803 by insertion of a kanamycin resistance gene cassette (Fig. 1). PCR analysis of genomic DNA revealed that the *sll1214* transformants always contained still a considerable amount of the wild-type *cyc* gene copies (Fig. 1A). Using growth media with glucose

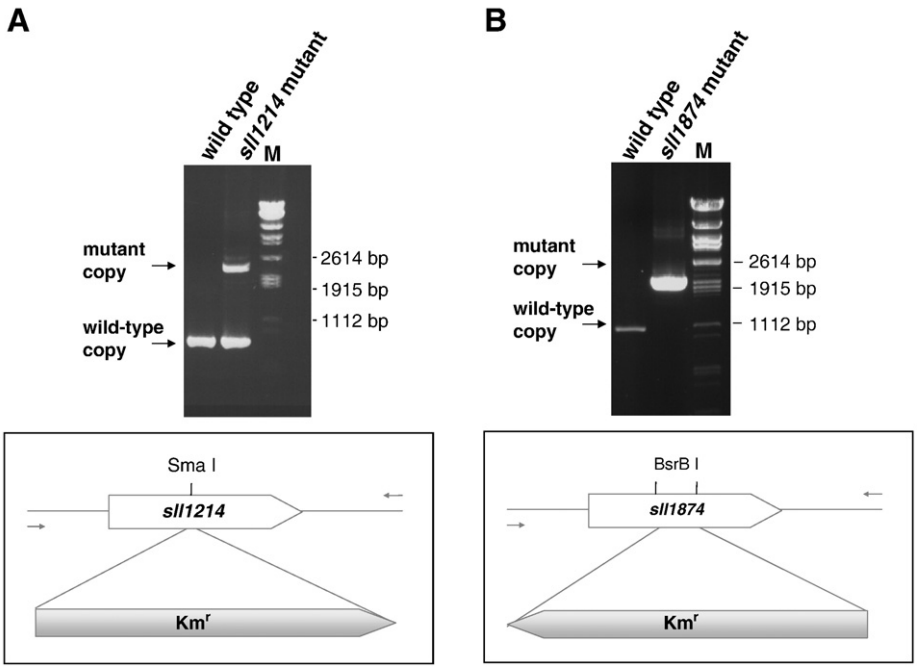

Fig. 1. Inactivation of the two *cyc* genes. PCR analysis of genomic DNA and scheme for the insertion of the resistance cassette. (A) For *cycI* a 1965 bp DNA fragment representing *cycI* together with the inserted resistance cassette and a shorter 415 bp DNA fragment representing the wild-type gene fragment were amplified. (B) PCR analysis of genomic DNA with *cycII*-specific primers revealed the complete segregation of the *cycII* knock-out mutant in comparison to the wild-type strain. It is suggested that under normal auto- and mixotrophic growth conditions *cycII* deletion can either be tolerated or compensated by *cycI* expression, while only slightly reduced levels of an intact *cycI* gene indicate an essential role of this gene product.

(mixotrophic growth) or increasing amounts of antibiotics (up to 120 μg ml^{-1} kanamycin) failed to select fully segregated *cycI* deficient lines, although the ratio of wild-type to mutant gene copies was lower under mixotrophic than under photoautotrophic growth conditions. Mutating *cycI* in the glucose-tolerant strain of *Synechocystis* sp. PCC 6803 also did not lead to fully segregated mutants (not shown).

We tested various aerobic conditions to select for complete segregation of the *cycI*$^-$ mutant. *cycI*$^-$ did not grow at high light intensities. The aerobically grown *cycI*$^-$ mutant lines tolerated best light exposure of 20 μmoles photons m^{-2} s^{-1} and media supplemented with 0.2% glucose. This condition was generally applied for the cyanobacterial cultures unless other conditions were indicated. Dark mixotrophic growth or growth conditions of iron and copper deficiency did not increase the ratio of mutant versus endogenous *cycI* gene.

Under ambient oxygen and selective growth condition the *sll1874* transformants replaced all wild-type gene copies with the mutant construct (Fig. 1B) indicating that *cycII* is not essential under aerobic growth conditions. For clarification the two mutants are specified by *cycI*$^-$ and Δ*cycII*.

First indications of putative diverse functions of CycI and CycII in chlorophyll biosynthesis were observed, when the growth rates of both mutants were compared with that of the wild-type strain. The *cycI*$^-$ lines grew slower under normal aeration reaching 77% of the wild-type growth rate whereas the Δ*cycII* lines exhibited a growth rate of 110% in comparison to wild type. The pigment analysis in Table 1 indicates that *cycI*$^-$ had only around 64% of wild-type chlorophyll content and Δ*cycII* and wild type accumulated similar chlorophyll amounts under identical growth conditions (Table 1). The phycocyanin content of *cycI*$^-$ gave rise to 89% of the wild-type content, while Δ*cycII* contained the same amount as wild-type cultures (Table 1).

The amounts of pigments measured in aerobically grown wild-type and mutant cells corresponded to the whole cell absorption spectra shown in Fig. 2. When the absorption spectra were normalized to the phycocyanin maximum at 635 nm, *cycI*$^-$ cells possessed the lowest peak at 680 nm for chlorophyll. Thus, it is confirmed that the *cycI*$^-$ knock down mutation of aerobically grown cultures caused reduced accumulation of chlorophyll in comparison to the Δ*cycII* knock-out mutation and wild type.

3.2. Expression of cycI and cycII

Due to the differences in requirement of the two genes *cycI* and *cycII* under normal growth conditions, we determined their transcript and proteins levels. In consistency to [17] semi-quantitative RT-PCR analysis of both transcript levels of wild-type cells revealed that the corresponding amplicons for the *cycII* expression were visible after 32 amplification cycles, while *cycI* corresponding amplicon appeared 4 cycles earlier (Fig. 3A). This indicates for our wild-type strain at given growth condition that *cycII* mRNA accumulates at least 16-times less than *cycI* mRNA. The low transcript abundance of *cycII* made Northern blot analysis impossible. Photoautotrophically grown wild-type cultures showed a remarkable reduction of the *cycI* transcript

Table 1
Pigment contents of *Synechocystis* wild-type (WT) and mutant cultures in a BG-11 medium.

	WT	*cycI*$^-$	Δ*cycII*	*cycI*$^-$/FLAG-CycI	*cycI*$^-$/FLAG-CycII
Chlorophyll [μg mg $protein^{-1}$]	26.6 ± 2.9	16.9 ± 0.8	28.4 ± 1.6	29.1 ± 1.3	21.8 ± 2.1
Phycocyanin [μg mg $protein^{-1}$]	155 ± 20	138 ± 24	154 ± 23	149 ± 6	131 ± 16
Protoporphyrin IX [pmol mg $protein^{-1}$]	12.2 ± 9.8	25.3 ± 3.4	10.4 ± 3.0	37.7 ± 3.8	15.7 ± 3.0
Mg-protoporphyrin IX [pmol mg $protein^{-1}$]	0.89 ± 0.05	2.17 ± 1.00	1.26 ± 0.14	0.87 ± 0.09	4.03 ± 0.45
Mg-protoporphyrin IX monomethylester [pmol mg $protein^{-1}$]	7.1 ± 1.8	1057 ± 66	1.7 ± 0.9	10.2 ± 1.7	661 ± 52

The strains were grown at 30 °C in a BG-11 medium containing 0.2% glucose under continuous irradiance of 20 μmol photons m^{-2} s^{-1} in rotating Erlenmeyer flasks for ambient air conditions. Samples for analysis of pigments and chlorophyll precursors were harvested after two days.

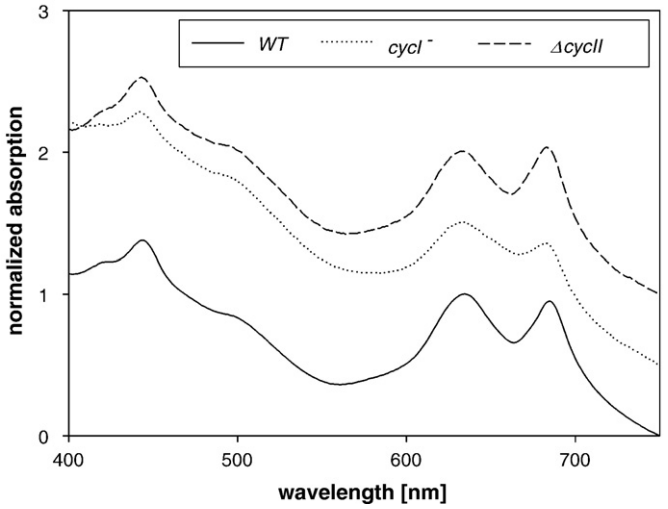

Fig. 2. Whole cell absorption spectra of *cycI*$^{-}$, Δ*cycII* and wild-type cells grown under low light intensities with 0.2% glucose. Peaks at 635 and 682 nm are due to phycocyanin and chlorophyll absorption, respectively. Spectra were normalized to phycocyanin absorption maxima at 635 nm and shifted for better visualization.

levels after 2 h incubation in darkness and high light intensities (Fig. 3B). These data derived from Northern blot hybridization are similar with previous microarray studies [28].

An immune-reacting protein band corresponding to the cyclase protein accumulated around 25% less in *cycI*$^{-}$ in comparison to the wild type (Fig. 4), while comparative semi-quantitative RT-PCR analysis of *cycI*$^{-}$ and wild-type transcripts revealed hardly any alteration of the *cycI* mRNA (Fig. 3A). Since *cycI*$^{-}$ did not accumulate more *cycII* transcript than the wild-type strain, a mutual compensatory up-regulation of the *cycII* gene could not be observed. In Δ*cycII* cells the *cycII* mRNA was not detectable (Fig. 3A) and the same amount of immune-reacting cyclase protein was found in comparison to wild-type cells (data not shown).

Western blot analysis (Fig. 4) indicated no significant alteration in the content of other proteins, which are also involved in tetrapyrrole biosynthesis in close proximity to cyclase, such as the ChlD, ChlI and ChlH subunits of Mg chelatase, the Mg protoporphyrin IX methyltransferase (ChlM) and the ferrochelatase (HemH). The anti-AtpB antibody was applied as loading control to indicate equal loading of wild-type and mutant protein extracts.

3.3. Metabolic consequences of cycI and cycII deficiencies on the Mg porphyrin-synthesizing pathway

Cells grown at 20 μmoles photon m^{-2} s^{-1} on glucose-containing medium were harvested for the analysis of chlorophyll intermediates (Table 1). Δ*cycII* accumulated similar amounts of Proto, MgProto and MgProtoME in comparison to the control strain. The *cycI*$^{-}$ cells accumulated 150 times more MgProtoME, the substrate for the cyclase reaction, than wild-type cells. The MgProto and Proto contents of *cycI*$^{-}$ were always two times higher than those of wild type indicating retention in the metabolic flow. It cannot be excluded that cultivation of *cycI*$^{-}$ is inhibited at high light conditions by the photo-oxidizing effects of accumulating MgProtoME and is likely already perturbed under low light and mixotrophic growth condition.

The reduced cyclase activity in the *cycI*$^{-}$ mutant could perturb the whole tetrapyrrole biosynthetic pathway. We measured from cell cultures of *cycI*$^{-}$ and wild type the synthesis rate of ALA and performed in vitro enzyme assays for Mg chelatase and ferrochelatase, which compete both for the same tetrapyrrole intermediate Proto (Fig. 5). ALA synthesizing capacity of *cycI*$^{-}$ was reduced to almost 50% of wild-type activity. The *cycI*$^{-}$ Mg chelatase activity was not significantly reduced, while its ferrochelatase activity decreased by one third in comparison to wild-type cells. The enzyme activities of the *cycI*$^{-}$ extract did not correspond with detectable alterations of the protein content for the contributing proteins of both chelating reactions (Fig. 4).

3.4. Complementation of the cycI^{-} and the ΔcycII mutations under aerobic growth

For complementation studies of *cycI*$^{-}$ and Δ*cycII* we engineered *Synechocystis* sp. PCC 6803 mutant cells to express CycI and CycII

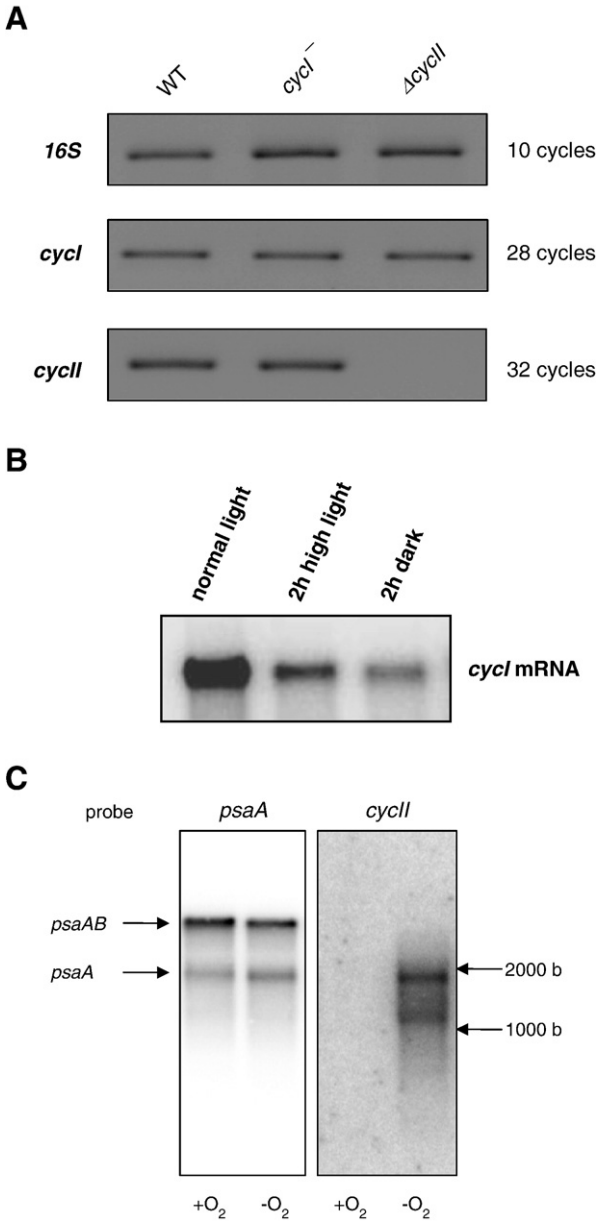

Fig. 3. Semi-quantitative analysis of the expression of the two genes encoding cyclases in *Synechocystis* sp. PCC 6803. (A) Non-saturating PCR was performed and stopped after the indicated number of cycles. 16S rRNA served as a control for performing reactions with equal quantities of RNA/cDNA. While *cycII* mRNA is completely absent in the corresponding mutant, the level of *cycI* mRNA is hardly reduced by the knock-down. Furthermore no up-regulation of the other *cyc* gene was detected in both mutants. Remarkably and indicative for its predominant role *cycI* mRNA is approximately 16 times more abundant than *cycII* transcript. (B) Expression of *cycI* is regulated by light. Both, high light intensities and darkness, led to a reduction of *cycI* transcript. (C) Northern blot analysis of *cycII* mRNA expression in wild-type cultures grown under aerobic and micro-oxic conditions used in this study. 3 μg of total RNA was separated, blotted and hybridized with *cycII* probe containing the whole *cycII* gene sequence. As a control a *psaA* DNA fragment was hybridized to the same RNA.

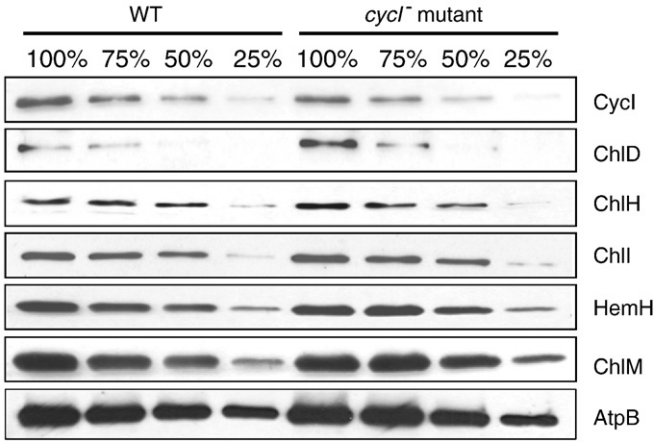

Fig. 4. Semi-quantitative Western blot analysis of enzymes involved in tetrapyrrole biosynthesis in *cycI*⁻ mutants in comparison to the wild type. Wild-type and mutant cell extracts equivalent to 25 μg protein (100%) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunodetection was performed using polyclonal antibodies raised against Chl27 from *Arabidopsis*, the ChlD, ChlH and ChlI subunits of the Mg chelatase complex, ferrochelatase (HemH), and MgProto methyltransferase (ChlM). An antibody raised against subunit AtpB of the F_0F_1-ATP-synthase was used as internal standard. Antibodies used for the immunoblots are indicated on the right side.

protein with an N-terminal fusion to 3×FLAG-Tag under the control of the *petJ* promoter. The *petJ* gene encodes cytochrome *c553* that replaces plastocyanin under copper deficiency in cyanobacteria. Preliminary experiments indicated no impaired chlorophyll biosynthesis and cyclase activity (indicated by determination of non-increased Mg porphyrin steady-state levels) during growth of wild-type strains under copper deficiency for more than two days. After transformation and stable insertion of these gene constructs into the wild-type and *cycI*⁻ genomes, expression of the recombinant CycI and CycII fusion proteins was monitored by Western blot analysis using an 3×FLAG specific antibody. We observed that the *petJ* promoter allowed low expression in a BG-11 medium, and thus, the FLAG-CycI and -CycII fusion proteins were already detectable prior to induction (Fig. 6A). The complementation tests were performed after 2 days of growth in the standard BG-11 medium. Since growth of *cycI*⁻ was compromised under aerobic conditions, complementation of the mutant strain was shown by comparing the absorption spectra of original and re-transformed cells and the analysis of chlorophyll, phycocyanin and the tetrapyrrole metabolites (Fig. 6B, Table 1). The absorption spectra of *cycI*⁻ cultures expressing FLAG-tagged CycI indicated a wild type-like ratio of the two absorbance peaks at 635 nm and 680 nm, the maxima for phycocyanin and chlorophyll-binding proteins, respectively (Fig. 6B), but upon expression of FLAG-CycII the absorption spectrum of *cycI*⁻ cultures did not alter to wild-type spectra. As the 680 nm absorption maximum tended to increase upon Flag-CycI expression in comparison to wild type we recorded the spectra of *cycI*⁻ cultures up to 8 days after induction under copper deficiency (Fig. 6C). The continuous increase at 680 nm relative to 635 nm indicates elevated chlorophyll contents. However, FLAG-CycI overexpression did not enable establishing of homozygous *cycI*⁻ mutant strains without the endogenous *cycI* gene.

The growth rate of *cycI*⁻ expressing FLAG-CycI reached around 100% of wild-type rate, while the *cycI*⁻ mutant strain expressing FLAG-CycII did not significantly change the growth rate in normal BG-11 media and yielded 70% of the wild-type rate, which was similar to that of the original *cycI*⁻ strain. It is obvious that the inability of complementation is not caused by an insufficient synthesis of FLAG-CycII fusion protein, because this protein was expressed even to higher amounts than the FLAG-tagged CycI counterpart (Fig. 6A).

The chlorophyll content in FLAG-CycI-expressing *cycI*⁻ cells reached wild-type levels, while the FLAG-CycII expression in the *cycI*⁻ background did not restore the wild-type chlorophyll content (Table 1). However, the content came up to wild-type levels after additional 11 days upon induced synthesis of FLAG-CycII (data not shown). The MgProtoME level was completely reduced in the aerobically grown FLAG-CycI expressing *cycI*⁻ strains, while the FLAG-CycII expressing *cycI*⁻ strain still accumulated huge amounts of this tetrapyrrole intermediate similar to the original *cycI*⁻ strain (Table 1).

3.5. Complementation of the cycI⁻ and the ΔcycII mutations under micro-oxic growth conditions

It was previously shown that Δ*cycII* hardly grew under micro-oxic conditions, while the same growth conditions enabled *cycI*⁻ deletion mutants to replace entirely the endogenous *cycI* gene [17]. Our semi-quantitative transcript analysis gave indication for a very low *cycII* expression under aerobic growth (Fig. 3A). Northern blot analysis of

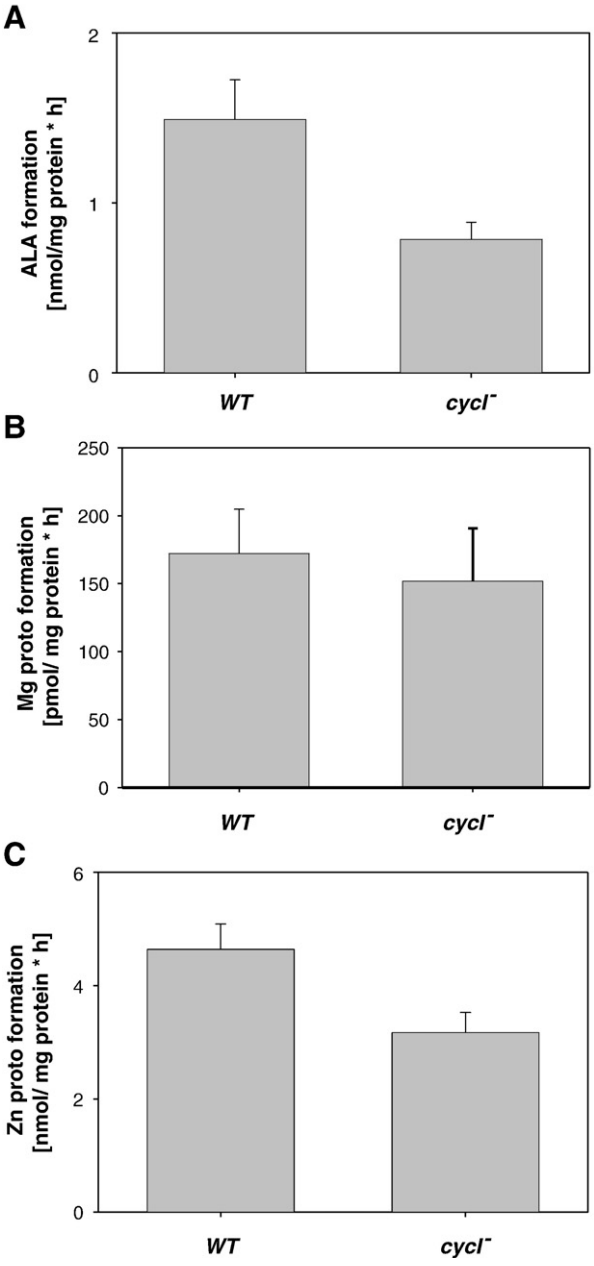

Fig. 5. Enzyme activities for key steps of tetrapyrrole metabolism were determined. While ALA synthesizing capacity (A) and Fe chelatase activity (B) were reduced in knock-down mutants of *cycI*, Mg chelatase activity was not adversely affected (C).

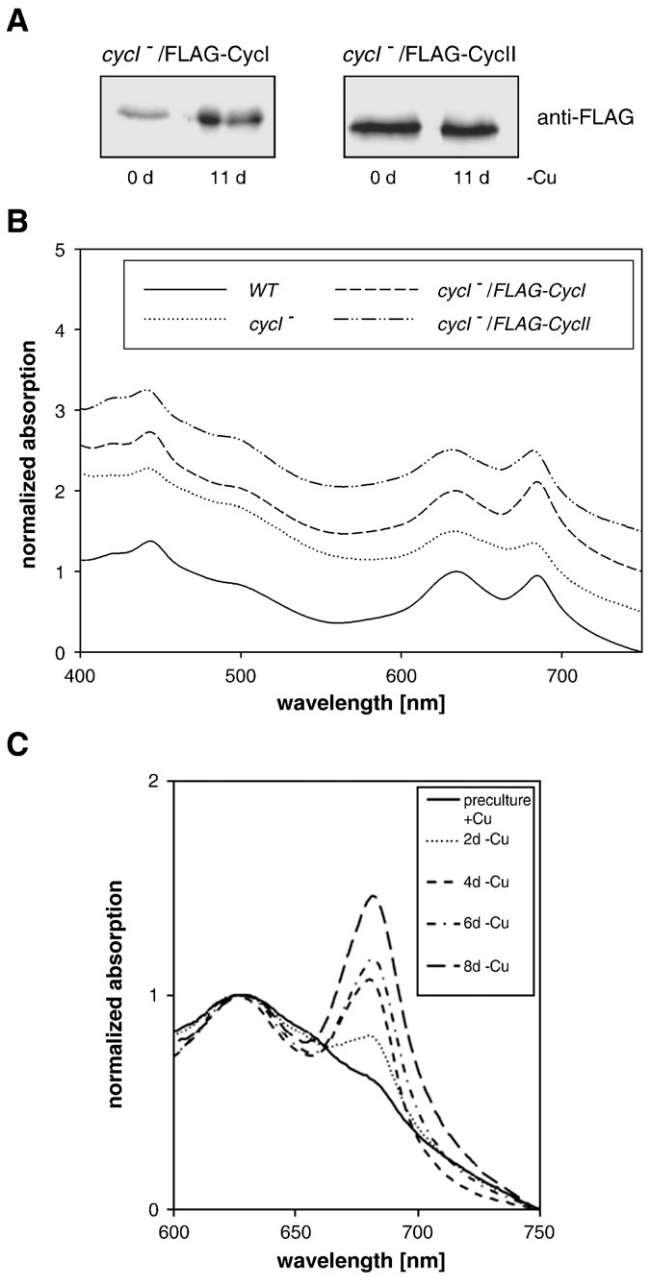

Fig. 6. Complementation analysis of *cycI*$^-$ mutant strain with FLAG-tagged CycI and CycII proteins under aerobic growth conditions (A) Western blot analysis revealed overexpression of FLAG-tagged cyclase proteins in *cycI*$^-$ knock-down mutants. Proteins were already expressed in detectable quantities before induction of expression of the recombinant proteins. (B) Whole cell absorption spectra of wild-type and mutant cells. Additional expression of a *FLAG-cycI* gene restores wild-type phenotype, whereas overexpression of FLAG-CycII did not compensate for the lack of CycI under normal growth conditions. Spectra were normalized to PC peak at 635 nm and shifted. (C) Whole cell absorption spectra of *cycI*$^-$/FLAG CycI mutant cells grown under prolonged copper limitation. Preculture was supplemented with 5 μM Cu^{2+} to repress FLAG-CycI expression from the *petJ* promoter.

anaerobically grown wild-type cells revealed two abundant transcripts of 1100 and 1900 nucleotides, which hybridized with a *cycII* probe (Fig. 3C). It is likely that the long transcript reflects a cotranscript of *cycII* and the coding sequences (*sll1875*) that is located downstream of *cycII* and encodes one of the two *Synechocystis* heme oxygenases.

We grew wild-type, *cycI*$^-$ and Δ*cycII* cultures under micro-oxic conditions with the aim to explore possible differences in the requirement and utilization of both cyclase isoforms under low oxygen conditions and to address the important question, whether CycII can complement *cycI*$^-$ deficiency under anaerobiosis. The cultures were grown under micro-oxic conditions for 2 days and harvested for comparative analysis of spectra of the cell cultures and the contents of chlorophyll and its metabolic precursors. The anaerobic growth conditions seemed to be disadvantageous for both *cycI*$^-$ and Δ*cycII* mutants. We confirmed that micro-oxic conditions impaired growth of the Δ*cycII* mutant cells. Microaerobically grown Δ*cycII* cells were characterized by an almost 40-fold increase of MgProtoME in comparison to wild-type cells (Table 2), while the MgProtoME content of the Δ*cycII* remained at a wild-type-like level when mutant cells grew under normal oxygenation (Table 1).

The cyclase reaction of *cycI*$^-$ cell cultures was also impaired under micro-oxic growth, because in the *cycI*$^-$ mutant cells 25-times more MgProtoME accumulated than in wild-type cells under the same culture condition (Table 2). In contrast to a previous report [17], growth of *cycI*$^-$ under micro-oxic conditions also failed to enforce the complete replacement of *cycI*$^-$ wild-type gene copies. Thus, it can be concluded that the chlorophyll-synthesizing pathway of both mutant strains is perturbed at the step of the cyclase reaction, when CycI protein content is reduced or CycII is missing under micro-oxic growth conditions. Contents of other chlorophyll intermediates were not remarkably modified in both mutant strains in comparison to wild-type cultures. However, it has to be mentioned that the deviation of porphyrin contents varied strongly in some biological replicates leading to high standard deviations. This holds true for Δ*cycII* and the merodiploid *cycI*$^-$ mutants. The latter strains contained a variable ratio of mutant to wild-type gene copies in different cultures, which led to gradually differing phenotypes with respect to chlorophyll deficiency and MgProtoME accumulation. Technical replicates showed only minimal deviations.

Complementation of the *cycI*$^-$ and Δ*cycII* mutations by the two FLAG-tagged cyclase proteins was shown in copper deficiency for two to three days (depending on the growth rate) under micro-oxic conditions by comparing the absorption spectra of original and re-transformed cells and analysis of the MgProtoME steady-state levels of the two mutants, *cycI*$^-$ and Δ*cycII* (Fig. 7, Table 2). Expression of the two FLAG-Cyc proteins was confirmed by Western blot analysis of anaerobically grown transformed *cycI*$^-$ cells (Fig. 7A). The FLAG-CycI expressing *cycI*$^-$ culture contained wild-type levels of chlorophyll and MgProtoME under oxygen deficient conditions. The full complementation was also confirmed by the absorption spectrum of *cycI*$^-$ cultures expressing FLAG-tagged CycI that displayed a wild type-like ratio of the two absorbance peaks at 635 nm and 680 nm (Fig. 7B). But, it is important to mention that the FLAG-CycII protein (similar to ambient oxygen conditions) does not compensate for CycI deficiency at micro-oxic growth conditions, because in CycI deficient cells expressing CycII high residual MgProtoME contents were detectable and the strain exhibited an absorption spectrum similar to the mutant cells (Tables 1 and 2, Fig. 7A). This is indicative for the highly sensitive response of the *cycI*$^-$ mutant strain at any tested growth condition, when endogenous *cycI* expression was compromised.

Table 2
Complementation analysis under micro-oxic conditions.

Mg-protoporphyrin IX monomethylester [pmol mg protein^{-1}]	
Wild type	6.7 ± 0.5
cycI$^-$	187.2 ± 115.5
Δ*cycII*	254.2 ± 57.1
cycI$^-$/FLAG CycI	6.6 ± 5.1
cycI$^-$/FLAG CycII	149.0 ± 84.3
Δ*cycII*/FLAG CycII	32.5 ± 12.1

For micro-oxic conditions aerobically precultured cells were further bubbled with N_2 in a fermenter for 2–3 days at 30 °C in a copper-deficient medium containing 0.2% glucose under continuous irradiance of 20 μmol photons m^{-2} s^{-1}.

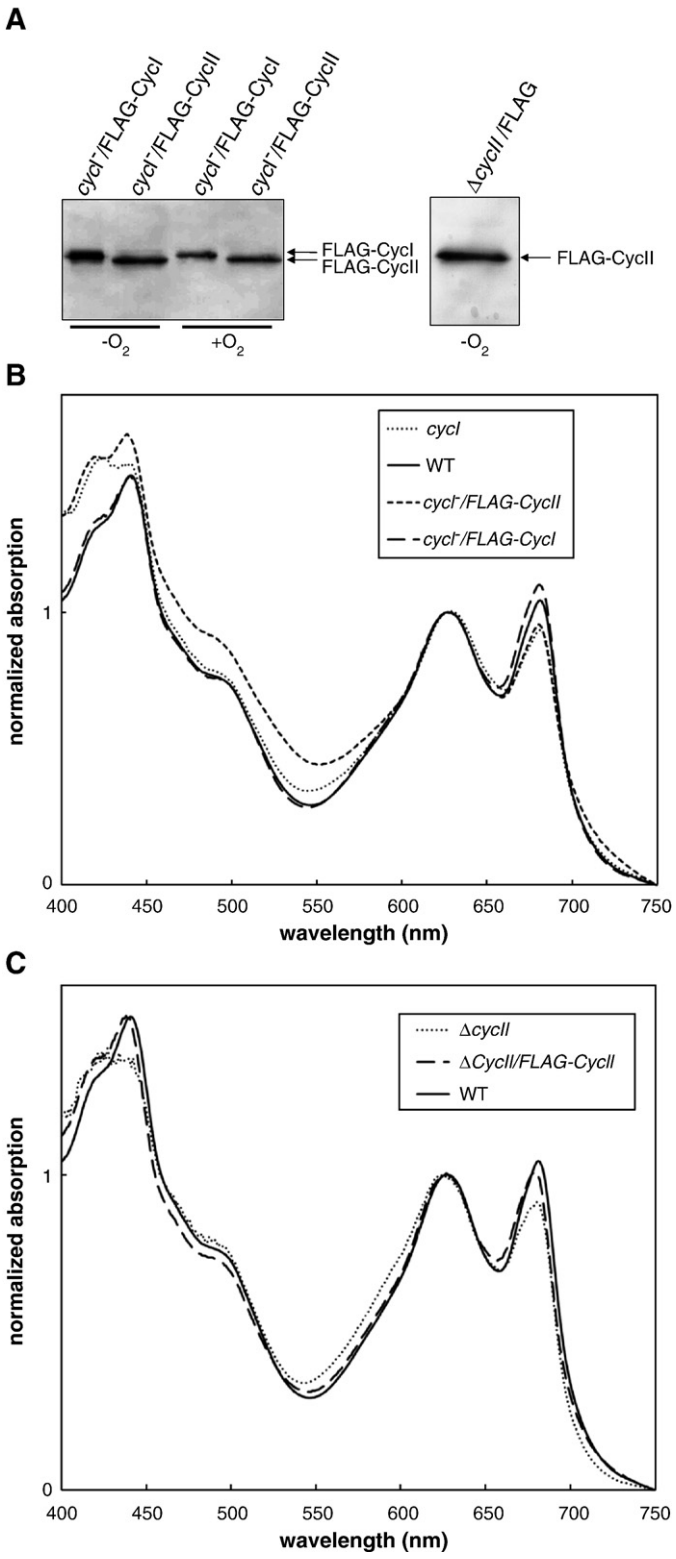

Fig. 7. Complementation analysis of *cycI*$^-$ and Δ*cycII* mutant strains with FLAG-tagged CycI and CycII proteins under micro-oxic growth conditions. (A) Western blot analysis of FLAG-CycI and FLAG-CycII expression under micro-oxic ($-O_2$) in comparison to aerobic growth conditions ($+O_2$) after 2 days of copper limitation. Proteins were detected using FLAG antibody. (B, C) Whole cell absorption spectra of wild-type and complemented *cycI*$^-$ (B) and Δ*cycII* (C) mutant strain grown under micro-oxic conditions. Spectra were normalized to phycocyanin absorption and optical density at 750 nm.

Additionally, the Δ*cycII* cultures expressing FLAG-CycII were analysed for complementation under micro-oxic growth conditions and their absorption spectrum and their MgProtoME steady-state levels were assayed. The high amounts of MgProtoME of the Δ*cycII* mutant under low oxygen growth were reduced by circa 90% after ectopic expression of FLAG-CycII at low oxygen for two days (Table 2). Continuous growth at microaerobic conditions further reduced the MgProtoME content to low wild-type levels. The absorption spectra of FLAG-CycII-expressing Δ*cycII* cell cultures resembled those of wild-type cultures indicating similar chlorophyll content under micro-oxic conditions (Fig. 7C). This indicates that CycII overexpression in Δ*cycII* cells restored full cyclase activity at low oxygen pressure and allowed accumulation of wild-type like amounts of chlorophyll.

4. Discussion

4.1. Different phenotypes of cycI$^-$ *and ΔcycII mutants at ambient and low oxygen*

Both open reading frames *sll1214* and *sll1874* encode an AcsF-like protein in *Synechocystis* sp. PCC 6803 with an unequivocal function in the cyclase reaction of the 5th ring formation of Mg porphyrins. An entire *cycII* knock-out enabled aerobic growth of the mutant strain without any adverse effect, while micro-oxic conditions impaired the Δ*cycII* mutant strain in the cyclase reaction, although the strain continued to grow for at least two days (Table 2, Fig. 7).

cycI$^-$ cultures were compromised during growth at low light and on glucose and showed phenotypically reduced chlorophyll content, while this mutant strain did not tolerate high light intensities. Attempts to inactivate the *cycI* locus completely by insertion of a kanamycin resistance cassette failed. Even under high selective pressure for the mutant allele the *cycI* transcript level was hardly reduced. The amount of CycI could only be reduced to less than 75% of the wild-type level (Figs. 3A, 4A), although the chlorophyll content and ALA biosynthesis (measured at ambient air conditions) decreased to 60 and 50% of remaining wild-type values, respectively (Table 1, Fig. 5A). As *cycI* could not be entirely inactivated it is concluded that this gene product is indispensable for chlorophyll biosynthesis.

Moreover, neither *cycII* expression was induced in response to impaired *cycI* expression nor ectopically expressed CycII compensated for the reduced CycI content in the *cycI*$^-$ strain. The important role of CycI can be also predicted from its transcriptional regulation: *CycI* expression is rapidly repressed in darkness and under high light intensities (Fig. 3B). These findings indicate an essential contribution of CycI to the cyclase reaction and a rate limiting function for the overall metabolic flow towards chlorophyll synthesis by a feedback-controlled reduction of ALA synthesis under aerobic growth condition (Fig. 5).

Minamizaki et al. [17] showed that micro-oxic conditions restore the growth of the *sll1214* mutant and enabled the complete substitution of the endogenous gene by mutational insertion, while the same condition adversely affected a knock-out mutant of *sll1874*. In our experiments microaerobically grown *cycI*$^-$ mutant cells failed to segregate completely into a homozygous mutant line. It is assumed that these differences are due to different wild-type strains used or to variations of the micro-oxic growth conditions used in both research groups. Northern blot analysis shows that *cycII* mRNA is efficiently expressed under the conditions we used in our experimental setup (Fig. 3C).

4.2. Only the homologous gene complements the respective cyc mutation

To substantiate our predictions concerning the requirement and the possible functional redundancy of both cyclase isoforms we performed complementation assays. From the absorption spectra and the MgProtoME accumulation profiles it can be deduced that the two cyclase isoforms cannot equivalently complement the deficiency of the other cyclase under ambient and microaerobic growth conditions in light. Additional expression of FLAG-CycI normalizes the metabolic

flow in *cycI*$^-$ at ambient and low oxygen conditions (Figs. 6B, 7A; Tables 1, 2). Here, the MgProtoME contents of *cycI*$^-$ mutant cells were reduced to the wild-type level. The *cycI* deficiency cannot be compensated by endogenous and ectopical overexpression of CycII at normal and micro-oxic conditions. The limited compensatory potential of CycII cannot be explained by its insufficient expression (Fig. 7A).

Inversely, *cycII* deficiency only affects the cyclase reaction at low oxygenation, which can be sufficiently normalized by additional *cycII* expression. Although *cycI* is expressed under micro-oxic conditions, CycI cannot substitute a CycII deficiency under these conditions. It is suggested that CycII is required under micro-oxic conditions and likely acts as a cyclase isoform with a higher affinity to molecular oxygen. Although Δ*cycII* cultures grew under micro-oxic conditions, the accumulation of MgProtoME indicates that CycI apparently does not function adequately and is perhaps less active at low oxygen concentration.

But, it is obvious that sole expression of CycII at low oxygen is also not sufficient. A reduced content of CycI at micro-oxic conditions also led to an accumulation of enormous amounts of MgProtoME and revealed its essential contribution for the cyclase reaction at low oxygen, which cannot sufficiently be substituted by CycII. Since both isoforms cannot entirely substitute each other, it is suggested that both proteins contribute differently to the cyclase reaction at the different growth conditions tested. It is proposed that the correct stoichiometry of CycI and CycII is relevant for intact cyclase reaction under low oxygen.

4.3. Photodynamic action of accumulating Mg porphyrins

Growth under ambient air conditions revealed that *cycI*$^-$ grew slower than the wild-type strain, which is most likely explained by more than 150-fold accumulation of MgProtoME within the cells (Table 1). No secretion of MgProtoME into the medium was found. It is worth to mention that protein deficiency in the cyanobacterial Mg branch of tetrapyrrole biosynthesis leads to different extents of metabolite accumulation. The Δ*gun4* mutant showed reduced activities of Mg chelatase and ferrochelatase and accepted only a 5–10 fold intracellular accumulation of Proto. But this mutant secreted huge amounts of Proto into the medium [27] indicating a selective excretion mechanism for different chlorophyll precursors.

Consistent with [17] the mutants for both cyclase genes displayed significantly increased levels of photosensitizing MgProtoME under micro-oxic growth conditions (Table 2). However, it is still unclear to which extent different accumulating chlorophyll precursors cause intracellular photodynamic damage and modify the metabolism of tetrapyrrole biosynthesis. Reduction of uroporphyrinogen decarboxylase, coproporphyrinogen oxidase, protoporphyrinogen oxidase and ferrochelatase activities in transgenic tobacco plants by antisense RNA expression for the respective genes leads to different maxima of tolerated accumulation of the particular substrate of each enzyme: Uroporphyrinogen, coproporphyrinogen, protoporphyrinogen and Proto accumulated at most 500, 50, 15 and 10-folds, respectively, in transgenic lines [29–32]. It is likely that the hazardous effects of photoreactive porphyrins and Mg porphyrins depend on the cellular subcompartment, in which they accumulate and whether they are deposited bound to proteins, as free or aggregated molecules in the membranes [33].

Thus, we currently suggest that a combination of accumulating photoreactive MgProtoME and reduced cyclase activity is responsible for the decelerated synthesizing capacity at the beginning (ALA synthesizing activity) and at the branch point of the pathway (Fe and Mg chelatase activity, Fig. 5). The regulatory interdependency between late and early steps in the cyanobacterial tetrapyrrole pathway needs to be investigated in future. It can also not be excluded that a lower supply of chlorophyll for the assembly of photosynthetic pigment–protein complexes triggers a reduced metabolic activity in tetrapyrrole biosynthesis.

4.4. Conclusion

CycI is the main contributor to aerobic cyclase reaction. The mutant strains do not tolerate lack of CycI and small reductions in the CycI amounts limit the overall synthesis of chlorophyll by reducing ALA synthesis. Under ambient air and low oxygen CycII is not able to substitute CycI in the *cycI*$^-$ mutant strains. Moreover, the deficient cyclase reaction of Δ*cycII* under low oxygen condition can only substantially be compensated by CycII overexpression. Then, these complemented strains exhibit wild type-like MgProtoME levels and slightly attenuated normal chlorophyll contents. Further studies began to examine the catalytic activities of recombinant CycI and CycII proteins with the emphasis on the heterodimeric complex formation under ambient and low oxygen conditions.

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