The *gun4* gene is essential for cyanobacterial porphyrin metabolism

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Abstract Ycf53 is a hypothetical chloroplast open reading frame with similarity to the *Arabidopsis* nuclear gene *GUN4*. In plants, *GUN4* is involved in tetrapyrrole biosynthesis. We demonstrate that one of the two *Synechocystis* sp. PCC 6803 *ycf53* genes with similarity to *GUN4* functions in chlorophyll (Chl) biosynthesis as well: cyanobacterial *gun4* mutant cells exhibit lower Chl contents, accumulate protoporphyrin IX and show less activity not only of Mg chelatase but also of Fe chelatase. The possible role of Gun4 for the Mg as well as Fe porphyrin biosynthesis branches in *Synechocystis* sp. PCC 6803 is discussed.

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1. Introduction

Chloroplast genomes contain mainly genes involved in photosynthesis and housekeeping. The remaining genes include open reading frames of unknown function that are designated *ycfs* (hypothetical chloroplast open reading frames). No final conclusion can be drawn about these *ycfs* without functional analysis [1]. Some *ycfs* occur in all plastid genomes of higher plants and algae. Others are conserved only in a limited number of algal plastid genomes. In these cases, similar sequences have been identified in the nuclear genomes of plants. Most of these *ycfs* are also present in cyanobacteria, suggesting that their gene products have highly conserved functions. *Ycf53* shows sequence similarity to the *Arabidopsis* nuclear gene *GUN4*. So far, *GUN4*-related genes were found only in photosynthetic organisms: in the nuclear genomes of *Arabidopsis* and rice, in three red algal chloroplast and most cyanobacterial genomes.

The *Arabidopsis gun* (genome unregulated) mutants were identified by their modified *Lhcb1* gene expression under norflurazon treatment and are characterized by deregulated communication between plastids and nucleus [2]. *Gun4* carries a nuclear mutant gene, which encodes a putative regulatory protein that interacts with Mg chelatase and stimulates its activity [3]. Mg chelatase is a highly regulated tetrapyrrole biosynthesis enzyme which catalyzes insertion of Mg^{2+} into protoporphyrin IX (Proto) and thus, directs Proto into the chlorophyll (Chl) synthesizing pathway [4]. Mg chelatase is a protein complex consisting of three subunits, CHL I, CHL H and CHL D of unknown stoichiometry [5]. Mutation in one of the *Arabidopsis* subunits or deregulation of the expression of tobacco CHL I and CHL H leads to Chl deficiency, but not to accumulation of the Mg chelatase substrate Proto [6,7]. Nuclear gene expression was deregulated in these mutants as a result of reduced Mg chelatase activity and modified Mg porphyrin levels, suggesting a direct transfer of information on metabolic activities of the plastid-localized pathway to the nucleus [6,8]. Mg porphyrin-dependent alteration of *Lhcb1* gene expression was observed in *Arabidopsis gun4* and *gun5* mutants [2,7,9].

In the current study, two of the *ycf53* genes in *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis* 6803) were inactivated to elucidate the function of the encoded proteins in tetrapyrrole biosynthesis, namely at the branch point directing Proto into either the Mg or the Fe branches in cyanobacteria.

2. Materials and methods

2.1. Culture conditions

Synechocystis 6803 wild-type (WT) and mutant strains were grown at 30 °C in BG-11 medium [10] under continuous irradiance of 30 μmol photons m^{-2} s^{-1} with air bubbling or in rotating Erlenmeyer flasks. The WT strain originates from the laboratory of S. Shestakov (Moscow, Russia). Transformants of *Synechocystis* 6803 were selected on media with increasing amounts of kanamycin (5 up to 40 μg ml^{-1}). Cultures were supplemented with filter-sterilized glucose (0.2%) when grown photoheterotrophically.

2.2. Mutagenesis

The *ycf53* genes (loci *sll0558* and *sll1380* [11]) were amplified using the primers P1 (5′ CTTCCGCTGGATCACCTTTA 3′), P2 (5′ GGTAATGACTTCCCCGAAGA 3′), P3 (5′ TTGCCTTAGATTGCCTTAGATTG 3′) and P4 (5′ AATAATTCATCCGGCAGACTGT 3′) (Fig. 1). PCR products were cloned into the pGEM-T vector (Promega, Mannheim, Germany). The kanamycin resistance cassette from pUC4K (New England Biolabs, Frankfurt/Main, Germany) was ligated into a single *Sma*I site for the *sll0558* construct and into a single *Hin*dII site for the *sll1380* inactivation construct. The constructs were used to transform *Synechocystis* 6803 as described [12]. Transformants were restreaked at least six times and analyzed by Southern

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Abbreviations: Chl, chlorophyll; Gun, genome unregulated; HPLC, high-performance liquid chromatography; LAHG, light-activated heterotrophic growth; MgProto, Mg-protoporphyrin IX; MgProtoME, Mg-protoporphyrin IX monomethyl ester; MgPMT, Mg-protoporphyrin IX monomethyl ester transferase; PC, phycocyanin; PS I, photosystem I; PS II, photosystem II; Proto, protoporphyrin IX; WT, wild type; ZnProto, Zn-protoporphyrin

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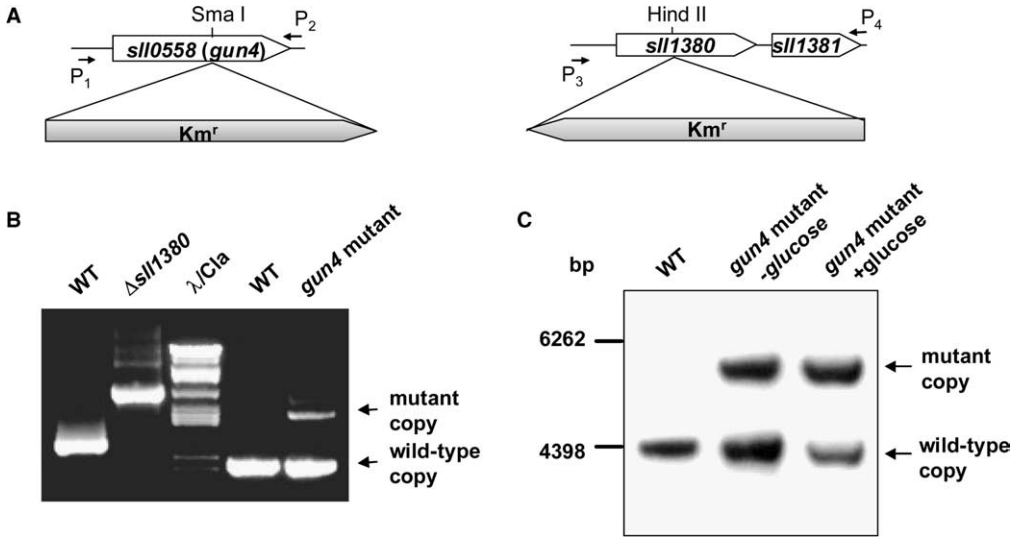

Fig. 1. Inactivation of *ycf53* genes in *Synechocystis* 6803. (A) The strategy for constructing donor plasmids with inactivated *sll0558* (*gun4*) and *sll1380* gene loci is shown. P1, P2, P3 and P4 are the primers used to verify complete segregation of the mutant strain. Km^r, kanamycin resistance cartridge (shaded boxes). (B) PCR amplification of the *gun4* and *sll1380* genes using total chromosomal DNA from WT and mutants as templates. (C) Southern blot analysis of genomic DNA isolated from WT and *gun4* mutant grown under photoheterotrophic and photoautotrophic conditions. The inactivated *gun4* gene gave rise to a larger *Nco*I fragment as compared to the native gene due to the Km^r inset.

hybridization and PCR to detect the level of segregation of WT genome copies.

2.3. DNA isolation and hybridization procedures

Genomic DNA of *Synechocystis* 6803 was extracted as described [13]. After digestion by restriction endonucleases and electrophoresis, gels were blotted onto nylon membranes. DNA fragments were labeled with [α-^{32}P]dATP (Amersham, Braunschweig, Germany) by the random priming method. DNA gel blot hybridizations were carried out as described [14]. Chromosomal DNA of *Synechocystis* 6803 was restricted with *Nco*I and hybridized with a fragment, amplified by PCR using primers P1 and P2 (see Fig. 1). Completeness of *sll0558* and *sll1380* gene inactivation was verified by PCR with LA Taq (TaKaRa, Gennevilliers, France) using specific primers.

2.4. Absorption and 77 K fluorescence emission spectra

Absorption spectra of whole cells were measured at room temperature using an UVIKON 933 spectrophotometer (Kontron, Milano, Italy). Chl contents were measured in methanol (90%) extracts of whole cells according to [15]. Phycocyanin (PC) contents were determined in the soluble fraction of cell extracts [16].

77 K fluorescence emission spectra were recorded using a Fluorolog FL-112 spectrofluorimeter with a 1680 emission double monochromator (Jobin-Yvon, Longjumeau, France). Samples were dark adapted for 10 min, suspended in 70% (v/v) glycerol/growth medium to a final Chl concentration of 5 $\mu g\,ml^{-1}$ and immediately dropped into liquid nitrogen. Frozen pearls were transferred to a Dewar vessel mounted in the fluorimeter. Fluorescence was excited at 440 nm.

2.5. SDS–PAGE and Western blotting

Proteins were separated by SDS–PAGE [17], transferred onto nitrocellulose membranes and immunolabeled with specific antibodies. Signals were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

2.6. Determination of enzyme activities

Cell cultures (800 ml) were pelleted, disrupted with 0.3 g glass beads in 3–5 ml cold 50 mM tricine, pH 8.0, 5 mM $MgCl_2$ and 1 mM DTT and subdivided for assays of Mg chelatase, Mg-Proto monomethyl ester transferase (MgPMT) and Fe chelatase activity, which were performed according to [18–20] with optimized temperatures: MgPMT was assayed at 33 °C, Mg chelatase at 30 °C and Fe chelatase at 28 °C. The amounts of enzymatic products correspond to the respective activities: Mg-Proto (MgProto), Mg-Proto monomethyl ester (MgProtoME) and Zn-protoporphyrin (ZnProto) for the activity of Mg chelatase, MgPMT and Fe chelatase.

2.7. Determination of porphyrins

Steady-state levels of Mg porphyrins were analyzed at a cell density corresponding to an OD = 1 at 750 nm. Pelleted cells were resuspended in methanol and incubated on ice for 15 min. After a short centrifugation, supernatants were collected and the pellets resuspended in methanol:acetone:0.1 N NH_4OH (v/v/v 10:9:1). Supernatants were combined after centrifugation at $13\,000 \times g$ for 5 min and prepared for high-performance liquid chromatography (HPLC) analysis. Extracted porphyrinogens were oxidized by addition of 5 μl of 1 M acetic acid and 5 μl of 2-butanone peroxide per 200 μl extract and separated by HPLC (Agilent-1100, Agilent, Waldborn, Germany) on a RP 18 column (Novapak C18, 4 μm particle size, 3.9×150 mm; Millipore, Eschborn, Germany) at a flow rate of 1 $ml\,min^{-1}$. Porphyrins were eluted with a linear gradient of solvent B (90% methanol and 0.1 M ammonium acetate, pH 5.2) in solvent A (10% methanol and 0.1 M ammonium acetate, pH 5.2) as follows: 0–100% in 7 min followed by 100% solvent B for 17 min. The eluate was monitored by fluorescence detection. The excitation and emission wavelengths were: for Proto 405 and 625 nm; for ZnProto 416 and 589 nm, for MgProto and MgProtoME 420 and 595 nm, respectively. Porphyrins were identified and quantified using authentic standards (Fluka, Germany; Porphyrin Products, UT).

3. Results and discussion

3.1. Inactivation of two ycf53 genes in Synechocystis 6803

Sequence analysis using the Blast-P algorithm [21] revealed three proteins with homology to the *Arabidopsis GUN4* gene product in *Synechocystis* 6803. The gene product of locus *sll0558* showed the highest similarity to *Arabidopsis* GUN4 (25% amino acid identity), whereas the other two *Synechocystis* 6803 genes (*sll1380* and *slr1958*) were less similar (22% and 20% amino acid identity, respectively). At least one *GUN4*-related gene was also found in the chloroplast genomes of the red algae *Porphyra purpurea*, *Cyanidium caldarium*, *Cyanidioschyzon merolae* and in all cyanobacterial genomes sequenced so far, except *Gloeobacter violaceus* PCC7421. The

two *ycf53* genes *sll0558* and *sll1380* with the highest similarity to *Arabidopsis GUN4* were inactivated in *Synechocystis* 6803 by insertion of a kanamycin resistance gene cassette (Fig. 1A). The *sll0558* transformants are designated *gun4* mutants. Only *sll1380* mutant cells replaced all WT copies with the mutant allele (Fig. 1B), whereas considerable levels of WT copies were detected in all *gun4* mutant clones that were tested over a period of 6 months. Analyses of genomic DNA isolated from *gun4* mutant cultures grown under photoheterotrophic and photoautotrophic conditions are shown in Fig. 1C. A 5900-bp fragment representing the mutant gene copy and a second hybridizing band of 4400 bp (the WT gene) were detected in *Nco*I digests of the mutant DNA samples. Photoheterotrophically grown *gun4* mutant cells contained genomic DNA with a higher portion of the 5900-kb mutant gene fragment than the photoautotrophically grown cultures (Fig. 1C). *Gun4* mutants obviously tolerate more mutant gene copies under photoheterotrophic conditions. The relative ratio of WT to mutant gene copies was 1:1.8 under photoheterotrophic conditions in contrast to 1:0.8 under photoautotrophic conditions, indicating an essential role of *gun4* for survival of cyanobacterial cells.

3.2. Phenotypic analysis of ycf53 mutants

Sll1380 mutant cells had only slightly reduced doubling times when compared to the WT. Contents of PC and Chl were not significantly altered in *sll1380* mutant cells in comparison to WT (data not shown). In contrast, *gun4* mutant cells differed clearly from the WT by their distinct blue color. *Gun4* mutants showed a significant increase in PC absorption at 625 nm (Fig. 2A). Pigment analyses revealed that Chl content was significantly reduced in the mutant accompanied by a slightly increased PC content (Table 1). 77 K fluorescence emission spectra were recorded to assess the impact of the *gun4* mutation on the photosynthetic apparatus in *Synechocystis* 6803 (Fig. 2B). Chl *a* fluorescence was excited at 440 nm, eliciting emission with maxima at 685, 695 and 725 nm. Whereas the former two maxima represent emission of photosystem (PS) II, the latter – dominant – emission peak derives from Photosystem I (PS I). The PS I-peak is significantly diminished in the *gun4* mutant spectrum, indicating a reduced amount of PS I (about 60% of the WT). A loss of Chl affects mainly PS I in cyanobacteria, since most of the Chl is associated with PS I [22,23]. Photosystem II (PS II) appears to be much less affected in the *gun4* mutant.

Chl-deficiency was more pronounced under photoheterotrophic conditions (Table 1). Under these conditions, mutant cells had only 61% of WT Chl content and 30% more PC. In contrast, under photoautotrophic conditions *gun4* mutant cells showed WT-like PC content and 73% of the WT Chl contents.

Growth rates of WT and *gun4* mutant cells were determined under photoautotrophic and photoheterotrophic conditions. Since limiting light (30 μmol photons m^{-2} s^{-1}) was used, the growth rate of photoautotrophically grown WT cells reached only 70% of that under photoheterotrophic conditions. The *gun4* mutant grew well under photoautotrophic conditions with no reduction of the growth rate in comparison to the WT. In medium supplemented with 0.2% glucose, mutant cells showed 81% of the WT growth rate. Under these conditions, a Southern blot analysis revealed a 1:1.8 WT-to-mutant gene copy ratio (Fig. 1C). Thus, growth inhibition of mutant cells and low Chl content under photoheterotrophic conditions correlate with lower WT gene copy number in comparison to photoautotrophic conditions. Even a partial mutation of *gun4* impairs cell viability. We suppose that a total knock-out of WT genes would be lethal.

It was shown that *Synechocystis* 6803 WT can grow heterotrophically under so-called light-activated heterotrophic growth conditions (LAHG, 5 min blue light per day) [24]. WT strains of *Synechocystis* 6803 cultivated in different laboratories, however, can differ substantially in physiological properties [25]. The WT used in this study is able to grow in complete darkness in contrast to the WT strain from the Pasteur Institute requiring LAHG conditions (data not shown). Remarkably, even in complete darkness the *gun4* mutant maintained a certain amount of WT gene copies. This may imply that

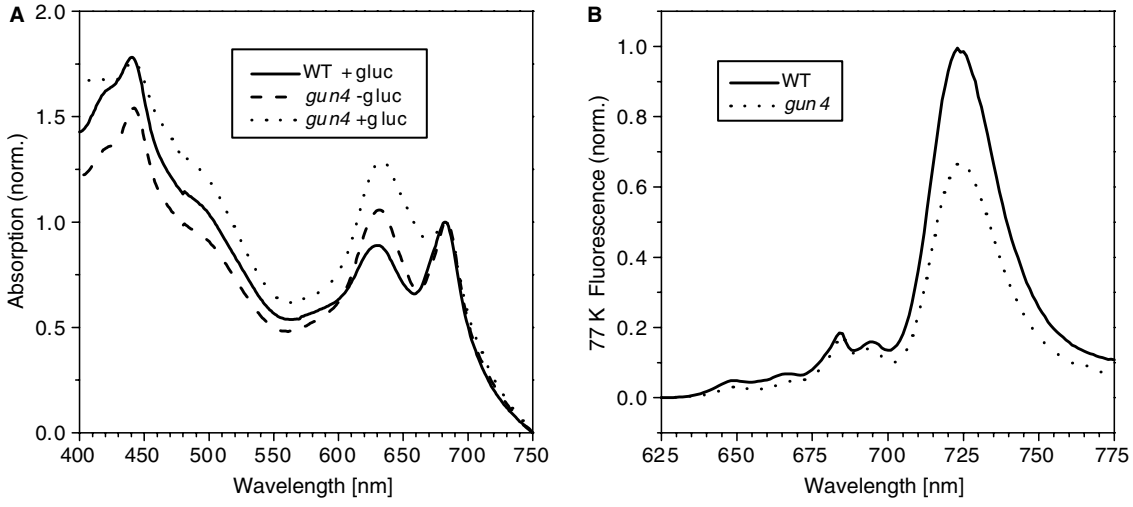

Fig. 2. (A) Absorption spectra of *Synechocystis* 6803 WT and the *gun4* mutant cells grown with or without glucose. Peaks at 440 and 682 nm are due to Chl *a*, and the peak at 625 nm is due to PC. Spectra were corrected for light scattering at 750 nm and normalized to Chl absorption at 682 nm. (B) 77 K fluorescence emission spectra of *Synechocystis* 6803 WT and the *gun4* mutant. Fluorescence was excited at 440 nm, spectra are normalized to their relative Chl *a* contents.

Table 1
Pigment content in WT and *gun4* mutant cells under photoautotrophic and photoheterotrophic growth conditions

Conditions	Strain	Chl content ($\mu g\,(mg\ protein)^{-1}$)	PC content ($\mu g\,(mg\ protein)^{-1}$)
Photoheterotrophic	WT	30.8 ± 5.1	218.4 ± 8.1
	gun4	18.7 ± 3.2	283.6 ± 26.8
Photoautotrophic	WT	29.4 ± 3.7	255.2 ± 17.3
	gun4	21.5 ± 4.2	264.3 ± 27.4

consequences of the *gun4* mutation are not restricted to Chl biosynthesis.

Gun4 mutant cells grown without kanamycin replaced most mutant gene copies by the WT gene within 5 days. In addition, the Chl content of mutant cells grown without kanamycin rose up to 94% of the WT level after 5–7 generations. These findings confirm that the mutant phenotype is indeed due to inactivation of the *gun4* gene rather than to compensatory mutations. However, polar effects of the kanamycin resistance cassette on genes located down- or upstream cannot be excluded.

3.3. Effects of the gun4 mutation on tetrapyrrole biosynthesis

In *Arabidopsis*, the GUN4 protein appears not to be essential for Chl synthesis under normal light exposure. However, GUN4 is apparently required for efficient Mg porphyrin synthesis [3]. A reduced *gun4* WT gene copy number renders the *Synechocystis* 6803 *gun4* mutant less competitive under photoauto- and photoheterotrophic conditions. This suggests that cyanobacterial Gun4 does not exclusively function in Mg chelation and supply of MgProto to Chl synthesis.

To further explore the function of Gun4 in *Synechocystis* 6803 Mg chelatase, MgPMT and Fe chelatase activities were determined in photoheterotrophically grown cells (Fig. 3A). The lower Mg chelatase activity of the *gun4* mutant in comparison to the WT is consistent with the idea that Gun4 promotes Mg chelatase–substrate interactions as shown in experiments using recombinant Mg chelatase [3]. MgPMT activity was reduced to a similar extent in the *Synechocystis gun4* mutant. The lower activity of both enzymes significantly impairs the metabolite flow in the Mg porphyrin branch as reflected by a reduced Chl content in the mutant (Table 1). Interestingly, *gun4* mutant cell extracts exhibited also less Fe chelatase activity than the WT (Fig. 3A). The enzyme activities in the Mg- and Fe porphyrin branches of the *gun4* mutant were reduced to 20–30% of WT activity (Fig. 3A). This would be expected to affect steady-state levels of porphyrins. Thus, contents of Proto, MgProto and MgProtoME in mutant and WT cells were determined (Fig. 3B). While the levels of MgProto and MgProtoME were not significantly altered, the *gun4* mutant contained 3–4 times more Proto than the WT.

A pivotal role of Gun4 at the branch point of tetrapyrrole biosynthesis may explain the requirement of a certain level of the Gun4 protein to sustain growth of *Synechocystis* 6803 cells. The *gun4* mutant tolerates a Chl reduction to only 61% of the WT level. Other cyanobacterial mutants can tolerate much lower Chl contents [26]. Hence, the function(s) of Gun4 appear to be more essential than mere optimizing of Mg porphyrin synthesis.

We suggest that complete deficiency of Gun4 would entirely interrupt tetrapyrrole biosynthesis. Reduced Gun4 levels affect Mg chelatase activity as well as the following enzyme, MgPMT, and more importantly, also Fe chelatase activity. Thus, the *gun4* mutant not only retains a certain substrate flow into the Mg porphyrin biosynthetic pathway, but it also suffers from lower protoheme formation. The inability to replace all WT gene copies by the mutant copy – with or without supply of glucose to the media – strongly corroborates the idea that Gun4 not only affects the Mg porphyrin branch, but is also essential for heme synthesis and, possibly, other metabolic pathways depending on it. A possible explanation for the higher amount of mutant gene copies found under photohet-

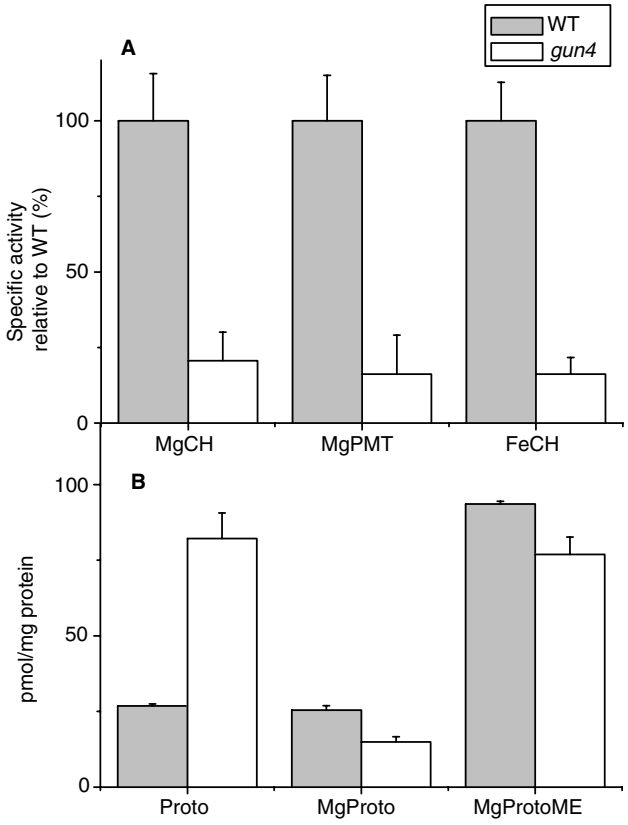

Fig. 3. Steady-state levels of intermediates and specific activities of tetrapyrrole biosynthetic enzymes in *Synechocystis* 6803 WT and *gun4* mutant cell lysates. (A) Enzyme activities of Mg chelatase (MgCH), MgPMT and Fe chelatase (FeCH) relative to WT. The absolute values for the enzyme activities in the WT are: MgCH: 589.5 ± 92.05 pmol mg^{-1} protein h^{-1}, MgPMT: 129.0 ± 19.31 nmol mg^{-1} protein h^{-1} and FeCH: 108.0 ± 13.7 pmol mg^{-1} protein h^{-1}. Values represent means ± S.D. of three different enzymatic reactions. (B) Total amounts of different porphyrin intermediates: Proto; MgProto and MgProtoME extracted from photoheterotrophically grown cultures.

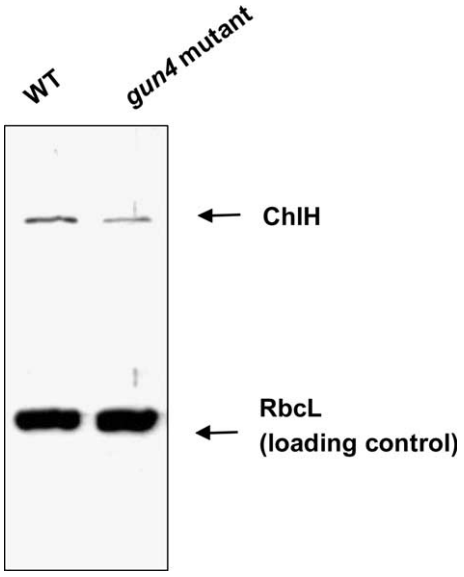

Fig. 4. Immunoblot of *Synechocystis* 6803 WT and *gun4* mutant protein extracts with an antibody directed against the ChlH subunit of Mg chelatase. Whole cell extracts corresponding to 35 μg protein were loaded in each lane.

erotrophic conditions might be the utilization of glucose by an alternative non-respiratory way of energy generation.

Two possible functions of Gun4 are conceivable: (i) Gun4 might be responsible for optimized Proto distribution to both chelatases and MgPMT. This role would be consistent with a binding capacity for Proto [3] and MgProto. (ii) Gun4 might be essential for stability of Mg chelatase and/or Fe chelatase. The reduced content of ChlH in *gun4* mutant cells (Fig. 4) could be indicative for a stabilizing role of Gun4 for the chelatase complex and is possibly responsible for the lower Mg chelatase and MgPMT activities. However, we cannot exclude that the Fe chelatase activity and/or stability was reduced in response to the lower enzymatic activities in the Mg porphyrin branch.

In conclusion, we have shown that *Synechocystis* 6803 Gun4 modulates the enzyme activities at the beginning of both, the Mg and Fe porphyrin branches of tetrapyrrole biosynthesis. This finding suggests that the function of Gun4 is not restricted to Mg porphyrin formation.

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