

Importance of the Cyanobacterial Gun4 Protein for Chlorophyll Metabolism and Assembly of Photosynthetic Complexes*

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Gun4 is a porphyrin-binding protein that activates magnesium chelatase, a multimeric enzyme catalyzing the first committed step in chlorophyll biosynthesis. In plants, GUN4 has been implicated in plastid-to-nucleus retrograde signaling processes that coordinate both photosystem II and photosystem I nuclear gene expression with chloroplast function. In this work we present the functional analysis of Gun4 from the cyanobacterium *Synechocystis* sp. PCC 6803. Affinity co-purification of the FLAG-tagged Gun4 with the ChlH subunit of the magnesium chelatase confirmed the association of Gun4 with the enzyme in cyanobacteria. Inactivation of the *gun4* gene abolished photoautotrophic growth of the resulting *gun4* mutant strain that exhibited a decreased activity of magnesium chelatase. Consequently, the cellular content of chlorophyll-binding proteins was highly inadequate, especially that of proteins of photosystem II. Immunoblot analyses, blue native polyacrylamide gel electrophoresis, and radiolabeling of the membrane protein complexes suggested that the availability of the photosystem II antenna protein CP47 is a limiting factor for the photosystem II assembly in the *gun4* mutant.

The three major tetrapyrrole end products chlorophyll (Chl),³ heme, and phycobilins are synthesized in a branched metabolic pathway with protoporphyrin IX (P_{IX}) as the last common precursor (1–4). The iron branch insertion of Fe²⁺ into P_{IX} by ferrochelatase leads to the formation of protoheme, whereas in the magnesium branch magnesium chelatase cata-

lyzes the chelation of Mg²⁺ into P_{IX}, thereby directing tetrapyrroles into Chl synthesis.

Cyanobacteria and plants accumulate various tetrapyrrole species in different quantities in the cell. Unlike plants, which use Chl-containing antenna complexes for light harvesting, the major light-harvesting antennae of cyanobacteria consist of phycobiliproteins, which contain covalently bound phycobilins originating from heme. Thus, in cyanobacteria P_{IX} might be directed into the iron branch to a much larger extent than in plants. Regulation of the P_{IX} distribution at the branch point between the magnesium and iron pathways as well as coordination of tetrapyrrole and cognate apoprotein synthesis are substantial in order to avoid accumulation of photoreactive intermediates during tetrapyrrole synthesis. For instance, in plants the genes coding for the light-harvesting Chl-*a/b*-binding proteins (*LHCB*) and for Chl biosynthesis proteins are simultaneously up-regulated in the light (5–7).

In a search for *Arabidopsis thaliana* mutants that are deregulated in the communication between plastids and the nucleus several *gun* (genome uncoupled) mutants have been identified (8). The *GUN2*, *GUN3*, *GUN4*, and *GUN5* genes encode proteins that are involved in tetrapyrrole biosynthesis (9, 10). Two of the gene products, *GUN2* (heme oxygenase) and *GUN3* (biliverdin reductase), function in heme degradation. *GUN5* encodes the ChlH subunit of the magnesium chelatase complex (9), whereas *GUN4* encodes a protein that interacts with magnesium chelatase (10). *GUN4*-related genes were found only in photosynthetic organisms. In plants *GUN4* either is encoded in the nucleus or retained in the chloroplast genome as is the case with several algae, where it was previously designated as *ycf53*.

All cyanobacterial genomes sequenced so far contain one or more *gun4*-related genes, with the exception of the ancient cyanobacterium *Gloeobacter violaceus* PCC7421. An *A. thaliana* *GUN4* mutant forms white-yellow tissue under standard conditions or pale green tissue in dim light (10). The *A. thaliana* Gun4 protein was co-purified with the ChlH subunit of magnesium chelatase and binds P_{IX} as well as magnesium protoporphyrin IX (MgP_{IX}) (10). *In vitro* enzyme assays revealed a dramatically increased efficiency of the enzymatic Mg²⁺ insertion into P_{IX} in the presence of Gun4, especially at low Mg²⁺ concentrations (10, 11). Accordingly, the crystal structures of Gun4 proteins from two cyanobacterial species bare a specific por-

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³ The abbreviations used are: Chl, chlorophyll; P_{IX}, protoporphyrin IX; PS, photosystem; DM, β -dodecyl maltoside; BN-PAGE, blue native PAGE; GT, glucose-tolerant; HPLC, high performance liquid chromatography; MES, 4-morpholineethanesulfonic acid.

phyrin binding site supporting a function of Gun4 in tetrapyrrole trafficking (11, 12).

In a previous report we described an incompletely segregated *gun4* mutant of *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803), arguing for substantial roles of Gun4 in cell metabolism (13). Here, we succeeded in generating a fully segregated *gun4* mutant strain in the glucose-tolerant (GT) *Synechocystis* 6803 wild-type background. The analysis of this strain revealed that the absence of Gun4 strongly decreased Chl biosynthesis and completely abolished photoautotrophic growth. These mutant properties correlated with a drastically impaired assembly of the two photosystems (PS). Among all Chl-binding proteins the accumulation of the PSII subunit CP47 was most strongly affected in the *gun4* mutant. The regulatory interaction between Chl synthesis and PS accumulation is discussed.

EXPERIMENTAL PROCEDURES

Culture Conditions—*Synechocystis* 6803 wild-type (GT) and mutant strains were grown at 30 °C in BG-11 medium (14) containing 5 mM glucose under continuous irradiance of 5 μmol of photons $\text{m}^{-2} \text{s}^{-1}$ (low light intensity) or 50 μmol of photons $\text{m}^{-2} \text{s}^{-1}$ (medium light intensity) in rotating Erlenmeyer flasks. For microaerobic growth, the strains were grown under medium light in a 1% CO_2 , 99% N_2 atmosphere. The GT strain originates from the laboratory of W. Vermaas (Arizona State University). Transformants of *Synechocystis* 6803 were selected on media with increasing concentrations of kanamycin (5 μg up to 40 $\mu\text{g} \text{ml}^{-1}$). The *gun4* mutant strain was prepared as described in Wilde *et al.* (13). Complete segregation of the mutants was confirmed by PCR using primers 5'-CTTC-CGCTGGATCACCTTTA-3' and 5'-GGTAATGACTTCCCAGAAGA-3'.

Construction of a *Synechocystis* 6803 Strain Expressing the 3 \times FLAG-tagged Gun4 Protein—The coding sequence of the *Synechocystis* 6803 *gun4* gene was amplified using the primers 5'-CATAATGTCTGATAATTTGACCGAACTCTCC-3' and 5'-AGATCTTTACCAACCGTATTGGGACC-3'. An NdeI site that overlaps with the ATG start codon of the *gun4* 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 \times FLAG peptide (Sigma-Aldrich) were hybridized, thus creating NdeI-compatible overhangs, and then ligated into the NdeI site of the pDrive-*gun4* construct. The resultant construct was verified by sequencing. Using NdeI and BglII restriction enzymes, a DNA fragment encoding the FLAG-tagged Gun4 was excised and then ligated into pSK9 vector for chromosomal integration under the control of the copper dependent *petJ* promoter (15), which supports low level expression in BG-11 medium. *gun4* mutant cells were transformed with the resultant vector pSKgun4FLAG. Transformants were selected on BG-11 agar plates containing 7 $\mu\text{g} \text{ml}^{-1}$ chloramphenicol. For strong expression of the fusion protein under the control of the *petJ* promoter, cells were grown for at least 7 days in BG-11 medium lacking copper in the trace metal mix.

Determination of Enzyme Activity—Cell cultures (800 ml) were pelleted and disrupted with glass beads in PBS, pH 7.5, and cell debris was removed by centrifugation. Samples were sub-

divided for assays of magnesium chelatase and ferrochelatase activities, which were performed according to Papenbrock *et al.* (16). A typical assay mix contained 1 mM dithiothreitol (DTT), 0.1% bovine serum albumin, 25 mM ATP, 20 mM MgCl_2 , and 100 μM P_{IX} for magnesium chelatase or 1 mM DTT, 1% Tween 80, 2.5 mM palmitic acid, 10 mM ZnSO_4 , and 50 μM P_{IX} for ferrochelatase. The reactions were started by the addition of P_{IX} dissolved in DMSO, and the assay mixtures were further incubated at 28 °C. Reactions were stopped after 0, 20, 40, and 60 min by the addition of an equal volume of methanol to the reaction mixture followed by freezing in liquid nitrogen. After the initial methanol extraction all samples were extracted twice with acetone, methanol, 0.1 N NH_4OH (10:9:1; v/v/v). Reaction products were quantified by HPLC using authentic porphyrin standards.

Determination of Porphyrins—Steady state levels of magnesium porphyrins were analyzed in the exponential growth phase of the cell culture ($\text{OD}_{750 \text{ nm}} \sim 0.5\text{--}0.9$). Pelleted cells were resuspended in methanol and incubated on ice for 15 min. After a short centrifugation, supernatants were collected, and the pellets were resuspended in methanol, acetone, 0.1 N NH_4OH (10:9:1; v/v/v). Supernatants were combined after centrifugation at $13,000 \times g$ for 5 min and prepared for HPLC analysis. Extracted porphyrinogens were oxidized by the addition of 5 μl of 1 M acetic acid and 5 μl of 2-butanone peroxide per 200 μl of 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, 0.1 M ammonium acetate, pH 5.2) in solvent A (10% methanol, 0.1 M ammonium acetate, pH 5.2) as follows: 0 to 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, respectively: 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). For quantification of P_{IX} precipitated in growth medium, 100 ml of culture at $\text{OD}_{750} \sim 0.4$ were filtered through 4- μm cellulose filters to collect precipitated pigments in growth media. The filter was frozen in liquid nitrogen, powdered in a 2-ml Eppendorf tube, and dissolved in 0.5 ml of alkaline acetone. Acetone was then extracted with 250 μl of hexane to remove Chl. P_{IX} was diluted 50 \times in DMSO and quantified spectrofluorometrically using an authentic standard (Sigma).

Radioactive Labeling of Cells and Preparation of Membranes—Radiolabeling of cells using a mixture of [^{35}S]Met and [^{35}S]Cys (Trans-label, MP Biochemicals, Irvine, CA; final activity, 400 $\mu\text{Ci ml}^{-1}$) was performed at 30 °C for 20 min under illumination with 125 μmol of photons $\text{m}^{-2} \text{s}^{-1}$ as described in a previous study (17). For preparation of membrane and cytosolic protein fractions, cells ($\text{OD}_{750} \sim 0.5$) were washed and resuspended in thylakoid buffer containing 25 mM Hepes, pH 7.4, 10 mM MgCl_2 , 5 mM CaCl_2 , and 20% glycerol. The cell suspension was mixed with glass beads and broken in a Mini Bead Beater, and the resulted homogenate was divided into two halves. To prepare a whole cell extract, the first part was solubilized by 1%

Chlorophyll Deficiency in *Synechocystis gun4* Mutant

β -dodecyl maltoside (DM) and centrifuged at $32,000 \times g$ for 10 min at 4°C to discard unbroken cells. The second part was centrifuged at $100,000 \times g$ for 30 min at 4°C , and the supernatant containing soluble proteins was transferred to a new tube. Pelleted membranes were washed 3 times in thylakoid buffer and resuspended in the same buffer containing 1% DM, and unbroken cells were discarded by centrifugation. To prove whether proteins are tightly bound to the membrane, an additional washing step with 2 M NaCl was applied.

Protein Analyses—Analysis of membrane proteins under native conditions was performed by Blue Native (BN)-PAGE using the buffer system of Schagger and von Jagow (18). The isolated membranes were resuspended in 25 mM MES/NaOH, pH 6.5, containing 10 mM CaCl_2 , 10 mM MgCl_2 , and 25% glycerol. The membranes were then solubilized with DM (DM:Chl = 40 for WT and 160 for *gun4*[−], respectively, w/w) and analyzed at 4°C in a 4–16% polyacrylamide gel. Protein composition of complexes was then assessed by electrophoresis in a denaturing 12–20% linear gradient polyacrylamide gel containing 7 M urea (19). The whole lanes from the BN gel were excised, incubated for 30 min in 25 mM Tris/HCl, pH 7.5, containing 1% SDS (w/v), and placed on the top of the denaturing gel; two lanes were analyzed in a single denaturing gel. Proteins separated in the gel were either stained by Coomassie Blue or transferred onto a membrane for immunodetection. One-dimensional SDS-PAGE was carried out as described by Jansch *et al.* (20). For immunodetection proteins were transferred from gels onto nitrocellulose or polyvinylidene difluoride membranes and immunolabeled with specific antibodies. The primary antibodies used in the study were raised in rabbits against (i) residues 58–86 of the spinach D1 polypeptide, (ii) residues 380–394 of barley CP47, (iii) whole recombinant Gun4 and ferredoxin proteins from *Synechocystis* 6803 overexpressed in *Escherichia coli* using the pQE80 expression vector (Qiagen) and purified with nickel-nitrilotriacetic acid-agarose. The antibodies against *Synechocystis* 6803 ChlH and ChlD subunits of the magnesium chelatase were kindly provided by Prof. Neil C. Hunter (University of Sheffield). Protein signals were visualized using the Immobilon Western (Millipore, Bedford, MA) or Supersignal West Pico (Pierce) chemiluminescence detection systems. Purification of 3 \times FLAG-tagged Gun4 from *Synechocystis* 6803 was performed from solubilized whole cell extracts as previously described (21).

Electron Microscopy—Cells were washed twice in 50 mM phosphate buffer, pH 7.2, and fixed in a solution containing 2% glutaraldehyde in 50 mM phosphate buffer for 2 h. This was followed by 3 washes in phosphate buffer and post-fixed in 1% OsO_4 in 50 mM phosphate buffer for 1 h. Finally, the cells were washed 3 times and embedded in 2% agarose. The segments of agarose with cells were dehydrated in isopropyl alcohol and embedded in Spurr's resin. Thin sections were cut with a diamond knife and post-stained for 30 min with 5% aqueous uranyl acetate followed by lead citrate for 80 s. The samples were examined with a Jeol 1010 electron microscope equipped with CCD camera MegaView III and image processing software Analysis (3.1.110).

Absorption Spectra and Pigment Measurements—Absorption spectra of whole cells were measured with a UV-2401 PC

spectrophotometer equipped with an integrating sphere (Shimadzu). Chl was extracted from cell pellets (10 ml, $\text{OD}_{750} \sim 0.5$) with 100% methanol, and its content was measured spectrophotometrically (22).

77 K Fluorescence Emission Spectra—77 K fluorescence emission spectra were measured in liquid nitrogen with an Aminco spectrofluorimeter (Spectronic Unicam) using membranes washed twice with excess of thylakoid buffer. 100 μl of the sample at Chl concentration of 14 $\mu\text{g ml}^{-1}$ were used for the measurement with 2 μM rhodamine as an internal standard. The spectra were normalized to rhodamine peak (573 nm) and multiplied by relative Chl:absorbance ratio for individual strains to indicate their Chl fluorescence per cell. 77 K fluorescence emission spectra were collected using excitation wavelength 435 nm to excite Chl_a.

RESULTS

Mutant Characterization—We had previously analyzed the role of *Synechocystis* 6803 Gun4 in tetrapyrrole biosynthesis using a *gun4* mutant strain that was not fully segregated under different growth conditions. These *gun4* mutant cells had exhibited lower magnesium and ferredoxin activities accompanied by the accumulation of P_{IX} , the substrate of both enzymes. In the present study we used a GT *Synechocystis* 6803 strain for mutational analysis. This different lineage of the original *Synechocystis* 6803 strain is widely used for the dissection and analysis of photosynthetic mutants. Fully segregated PSI, PSII, and Chl biosynthesis mutants could be generated in this genetic background (23, 24).

PCR analysis (Fig. 1) revealed that *gun4* mutant in the GT substrain of *Synechocystis* 6803 (hereafter designated *gun4*[−]) was completely segregated. In contrast to previous results obtained with the PCC strain of *Synechocystis* 6803 (13), no chromosomal wild-type copies of the *gun4* gene were detected. *Gun4* inactivation was confirmed by immunoblot analysis, demonstrating the complete lack of Gun4 in protein extracts from the mutant (compare Fig. 3A). Because Gun4-deficient cells lost the ability to grow photoautotrophically, all media were supplemented with 5 mM glucose to support the growth of the *gun4* mutant cells. At a medium light intensity (50 μmol of photons $\text{m}^{-2} \text{s}^{-1}$) *gun4* mutant cells grew slowly, lost more than 90% of Chl, and accumulated large amounts of P_{IX} (Table 1). In addition, P_{IX} also strongly accumulated in the growth medium leading to the formation of brown porphyrin aggregates. When these aggregates were separated from cells by filtration, more than eight times more P_{IX} was found in *gun4*[−] cells compared with the wild type (data not shown). In addition, mutant cells lost substantial amounts of thylakoid membranes (Fig. 2B) at medium light intensity. In contrast, at low light intensity (5 μmol of photons $\text{m}^{-2} \text{s}^{-1}$) growth of the *gun4* mutant on glucose (Table 1) was improved, its thylakoid structure (Fig. 2C) rather resembled that of wild type, and endogenous accumulation of P_{IX} was decreased (Table 1). However, even under low light conditions, the Chl content in the mutant was only about 25% that of the wild-type level (Table 1), and the content of the Chl precursor MgP_{IX} monomethyl ester was strongly reduced (Table 1). Similar effects were observed when the *gun4* mutant was grown under microaerobic conditions at

medium light intensity. Chl content in the mutant increased from 8 to 15%, and the cells contained more thylakoid membranes than under aerobic conditions (Fig. 2D). The negative influence of both light and aerobic conditions on the formation of thylakoid membranes and Chl contents in the *gun4* mutant suggests increased light sensitivity and photooxidative damage in the absence of Gun4 at medium light intensity. To prove whether oxidative stress contributes to the *gun4*⁻ phenotype even at low light intensities, the mutant was grown microaerobically under these light conditions. No further increase in Chl accumulation was observed compared with aerobic growth at low light, and the Chl content in the mutant remained at 25% that of the wild-type level. In addition, the amount of Chl also was not enhanced in *gun4* mutant cells, grown aerobically in the dark (data not shown). It was concluded that low light intensity

did not photosensitize the mutant cells and did not cause light-dependent oxidative stress. Thus, strains were grown at low light intensities for further experiments.

Localization of Gun4, Ferrochelatase, and Magnesium Chelatase in the Cyanobacterial Cell—Gun4 is proposed to function at the branch point of Chl and heme biosynthesis. To localize Gun4 and the two metal chelatases in cyanobacterial cells, whole cell extracts of wild-type and *gun4* mutant cells were fractionated. Proteins of the cytosolic and membrane fractions were separated by SDS-PAGE, blotted, and probed with antibodies. Fig. 3A shows that the ferrochelatase is tightly bound to the membrane, as there is no significant decrease in signal intensity after additional washing of the membranes or treatment with 2 M NaCl. In contrast, the ChlH subunit of magnesium chelatase was detected both in the soluble and in the membrane fractions, whereas the ChlD subunit of the magnesium chelatase appears to be exclusively located in the soluble fraction. Gun4 was mainly detected as a soluble protein and appeared in the membrane fraction only to a minor degree. The substantially lower signal of Gun4 in the membrane fraction after treatment with 2 M NaCl suggests peripheral association of this protein with membranes (Fig. 3A).

In the fully segregated *gun4* mutant no immune-reacting protein band was detectable using the anti-Gun4 antiserum (Fig. 3A). Interestingly, in contrast to an equal distribution of wild type ChlH subunit of the magnesium chelatase between soluble and membrane fractions, in the *gun4* mutant this protein seems to be localized mainly in the soluble fraction (Fig. 3A). Intriguingly, *gun4*⁻ cells contained unequivocally lower amounts of ChlH and ferrochelatase (Fig. 3B) than the wild type. This finding corroborates the decreased activities of both enzymes *in vivo* (Fig. 3C). Whereas the loss of ferrochelatase activity was proportional to the loss of protein, loss of magnesium chelatase activity could not entirely be attributed to the ChlH deficiency, supporting a direct role of Gun4 for retaining the enzyme activity (10).

Affinity Purification of 3×FLAG-tagged Gun4—A possible explanation for the decreased activity of both chelatases in the *gun4* mutant might be their physical interaction with Gun4. To identify and specify interacting protein partners of Gun4, *gun4* mutant cells were engineered to express a 3×FLAG-tagged Gun4 protein. For this purpose we used a vector harboring the *petJ* promoter, which is induced by copper deficiency and a platform for stable insertion of the construct into the *Synechocystis* 6803 chromosome. Absorption spectra of whole cells confirmed that expression of 3×FLAG-Gun4 complemented

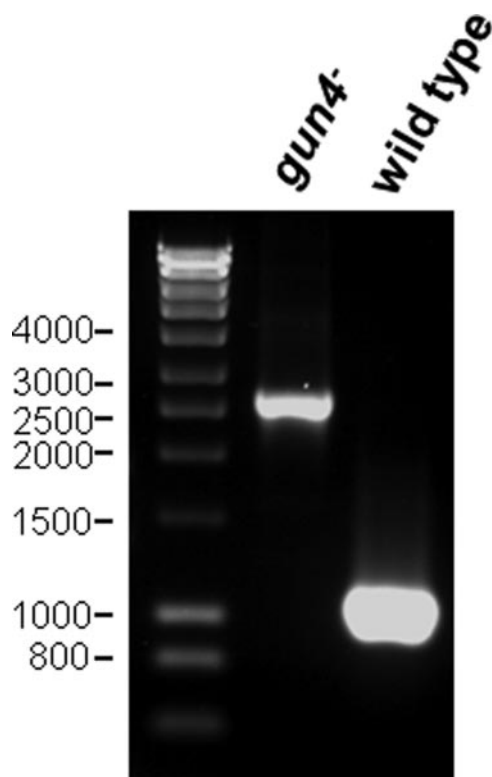


FIGURE 1. Inactivation of the *gun4* gene in *Synechocystis* 6803 (GT strain). Wild-type and mutant gene copies were amplified using total chromosomal DNA from wild-type and *gun4*⁻ strains as templates. PCR amplification of the *gun4* gene yielded a correct 1.1-kilobase pair product in the wild type. The mutant *gun4* copy gave rise to a larger PCR fragment of 2.6 kilobase pairs due to the insertion of the kanamycin resistance cassette (1.5 kilobase pairs). The sizes of the DNA marker fragments are indicated on the left.

TABLE 1
Phenotypical analysis of *gun4* mutant in comparison to the wild type

Values represent the mean ± S.D. of at least three independent experiments using different cultures harvested in logarithmic growth phase.

Strain	Low light intensity ^a		Medium light intensity ^b	
	Wild type	<i>gun4</i> ⁻	Wild type	<i>gun4</i> ⁻
Doubling time (h)	19 ± 0.5	21 ± 0.5	10 ± 0.2	25 ± 0.5
Chlorophyll (μg·mg protein ⁻¹)	11.3 ± 1.1	2.7 ± 0.2	11.8 ± 1.0	0.9 ± 0.4
Phycocyanin (μg·mg protein ⁻¹)	153 ± 36	154 ± 32	163 ± 45	131 ± 43
Protoporphyrin IX (pmol·mg protein ⁻¹)	17.2 ± 10.9	61.6 ± 51.2	4.7 ± 2.0	205.0 ± 100
Mg-protoporphyrin IX (pmol·mg protein ⁻¹)	1.17 ± 0.46	0.15 ± 0.06	1.00 ± 0.22	0.16 ± 0.06
Mg-protoporphyrin IX monomethyl ester (pmol·mg protein ⁻¹)	4.51 ± 0.87	0.98 ± 0.51	3.88 ± 1.12	0.72 ± 0.33

^a 5 μmol of photons m⁻² s⁻¹ in medium containing 5 mM glucose.

^b 50 μmol of photons m⁻² s⁻¹ in medium containing 5 mM glucose.

Chlorophyll Deficiency in *Synechocystis gun4* Mutant

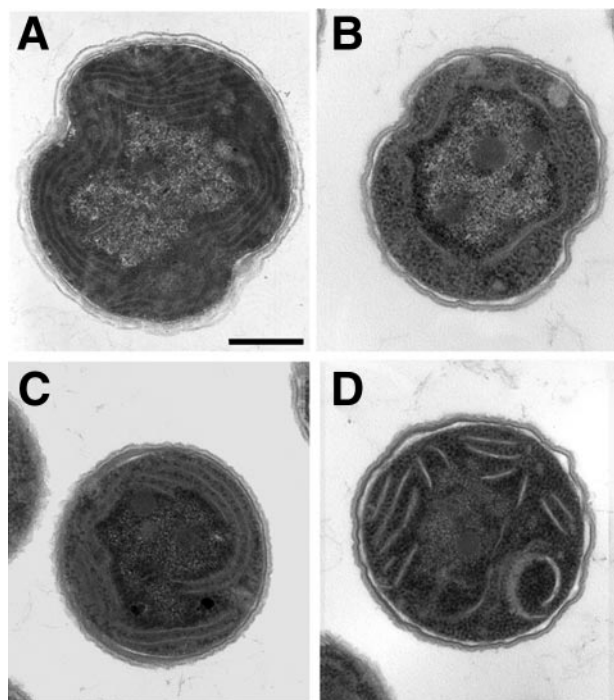


FIGURE 2. Transmission electron microscopy of *Synechocystis* 6803 cells. *A*, wild-type cells grown aerobically under medium light conditions. *B*, *gun4* mutant cells grown aerobically under medium light intensity. *C*, *gun4* mutant cells grown aerobically under low light intensity. *D*, *gun4* mutant cells grown microaerobically under medium light conditions. Scale bar = 500 nm.

the *gun4* mutation (Fig. 4A), indicating the generation and activity of a functional fusion protein. The 3×FLAG-Gun4 fusion protein was expressed during growth in copper-deficient medium and immunoprecipitated with anti-FLAG M2 affinity gel (Sigma-Aldrich) as described (21). The 3×FLAG-Gun4 protein was eluted by the addition of a 3×FLAG peptide and separated by SDS-PAGE (Fig. 4, C and D). Cells expressing the non-tagged Gun4 were used as the negative control. Among the proteins co-purified with Gun4, only ChlH was identified by immunoblot analysis (Fig. 4C). The other magnesium chelatase subunits ChlI and ChlD as well as ferrochelatase did apparently not associate with Gun4. Immunoblot analysis demonstrates that the magnesium chelatase subunits ChlH and ChlD as well as the ferrochelatase were present in similar amounts in cells expressing 3×FLAG Gun4 and control cells (data not shown). In addition, *in vitro* pulldown assays did not indicate an interaction between recombinant Gun4 and ferrochelatase (data not shown). No other putative interacting proteins were detected in the eluate fraction from the *gun4*⁻:*gun4*FLAG strain after affinity purification of the 3×FLAG-Gun4 protein (Fig. 4D).

Absence of *Gun4* Affects Accumulation of Both PSII and PSI—The drastically reduced Chl content in the *gun4* mutant (Table 1) was necessarily associated with lower amounts of the Chl-binding protein complexes PSII and PSI. Indeed, semi-quantitative measurements of 77 K fluorescence spectra of membranes using rhodamine as the internal standard revealed an almost complete loss of PSII complexes and a large decrease in the amount of PSI complexes in *gun4*⁻ cells compared with the wild type (Fig. 4B). The pronounced loss of PSII complexes

containing the CP47 protein (PsbB) was indicated by the absence of the 695-nm emission peak in *gun4*⁻, similar to the Δ *psbB* deletion mutant (25), which served as reference in this study (Fig. 4B).

The Chl deficiency in *gun4*⁻ gave us a unique opportunity to investigate consequences of very limited Chl supply for assembly and stability of Chl-binding proteins under standard light conditions. Therefore, we performed a detailed comparison of protein complexes of membranes isolated from wild-type and *gun4* mutant cells applying BN-PAGE (Fig. 5A). Apparently, on the per cell basis the contents of both PSI (RCCI) and PSII (RCCII) complexes were dramatically decreased in *gun4* mutant cells compared with wild-type cells (about 25% PSI and maximally 10% PSII of the wild-type level). The PSI trimer was the prevailing form in both strains, but the PSI trimer/PSI monomer ratio was apparently decreased in the mutant. In addition, the relative content of the dimeric form of PSII core com-

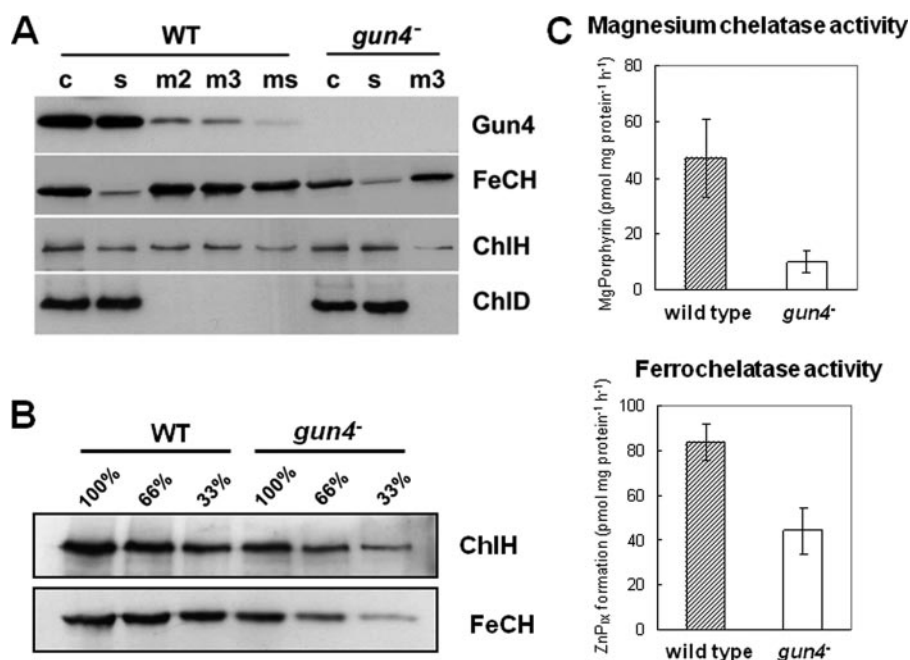


FIGURE 3. Localization, quantification, and activities of both chelataases. *A*, subcellular localization of Gun4, ferrochelatase (FeCH), and the magnesium chelatase ChlH and ChlD subunits. Membranes have been washed 2 (m2) or 3 (m3) times in thylakoid buffer followed by an additional wash with 2 M NaCl in thylakoid buffer (ms). Membrane fractions (m), crude (c), and soluble extracts (s) of wild-type and *gun4* mutant cells were separated by SDS-PAGE and transferred to nitrocellulose membranes. The amount of Chl corresponding to 150 μ l of cells at $A_{730} = 1$ was loaded per each lane. Polyclonal antibodies used for the immunoblots are shown on the right. *B*, accumulation of the ChlH subunit of magnesium chelatase and ferrochelatase in the crude cell extract were determined by immunodetection. Numbers indicate relative protein loading based on the amount of cells. *C*, activities of magnesium and ferrochelatase measured in wild-type and *gun4* mutant cells.

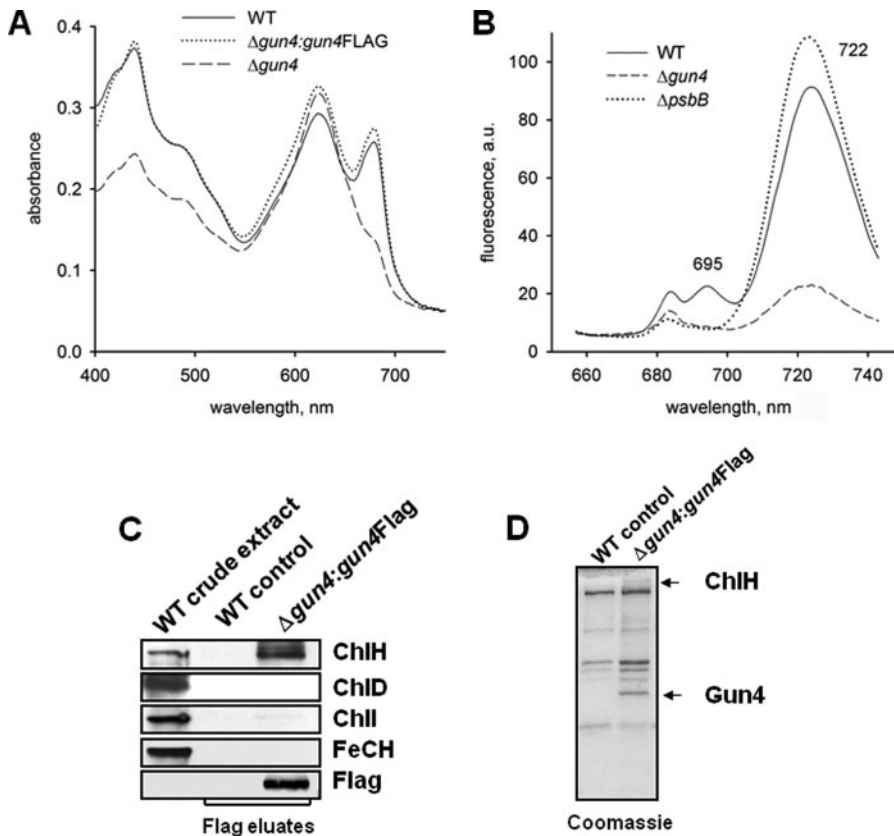


FIGURE 4. Phenotype of *gun4* mutant cells and affinity purification of Gun4. *A*, absorption spectra of *Synechocystis* 6803 wild-type (WT) and mutant cells lacking Gun4 (*gun4*⁻) or expressing a FLAG-tagged version of Gun4 (Δ *gun4*:*gun4*FLAG). Cells were grown with 5 mM glucose in the medium. Peaks at 620 and 682 nm represent phycocyanin and Chl absorption, respectively. Spectra of whole cells were measured with a UV-2401 PC spectrophotometer (Shimadzu, Kyoto, Japan). *B*, semiquantitative 77 K fluorescence emission spectra of thylakoid membranes from *Synechocystis* 6803 wild-type and mutant cells lacking Gun4 (Δ *gun4*) or CP47 (Δ *psbB*). Rhodamine was used as an internal standard, and the spectra were normalized to indicate Chl fluorescence per cell. Peaks at 685/695 and 722 nm represent PSII and PSI complexes, respectively. *a.u.*, absorbance units. *C*, affinity purification of 3 \times FLAG-Gun4 and identification of magnesium and ferredoxin (*FeCH*) subunits by immunodetection. Solubilized extracts of *gun4* mutant cells engineered to express 3 \times FLAG-Gun4 were immunoprecipitated with anti-FLAG M2-agarose (Sigma-Aldrich). FLAG-Gun4 complexes were eluted with 3 \times FLAG peptide, resolved by SDS-PAGE, stained with Coomassie (*D*) or blotted onto a nitrocellulose membrane and immunodetected (*C*). Wild-type eluate was used as a control.

plex (RCCII(2)) was lower in the mutant than in the wild type. The two-dimensional gel separation of proteins from wild-type membranes and the corresponding immunoblots revealed the presence of the CP47 protein in the core complex lacking CP43 (RC47) and in the region of unassembled proteins. Both free CP47 and CP47 integrated in RC47 were missing in thylakoids of *gun4*⁻. Moreover, the mutant accumulated two distinct complexes lacking CP47 previously designated as RC* and RCa (26). These complexes contain mature D1 or its partially processed form iD1, D2, cytochrome *b* 559, and PsbI and are typical for strains impaired in synthesis and/or stability of CP47 (17, 26). Thus, the results suggest that the insufficient accumulation of CP47 limits the assembly and accumulation of complete PSII core complexes. In contrast to the Chl-containing PSs, the ATP synthase was present in both strains in equal amounts showing that accumulation of membrane protein complexes lacking Chl was not changed after inactivation of the *gun4* gene.

The differential effect of the *gun4* deletion on accumulation of Chl-binding proteins was further supported by semiquantitative immunoblot analysis (Fig. 6). Consistent with the BN-

PAGE data, the ATP synthase was present in similar amounts in wild type and *gun4*⁻, whereas the amount of the PSII protein PsaD corresponded to about 25% that of the wild-type level. Among the PSII proteins, CP43 accumulated to 25% that of the wild-type level, whereas the D1 protein and especially CP47 were hardly detectable. Negligible cellular contents of CP47 corresponded with a non-detectable oxygen-evolving capacity of PSII, and no variable fluorescence was observed in the *gun4* mutant (not shown).

Because two-dimensional BN/SDS-PAGE was performed with thylakoids isolated from radioactively labeled cells, the analysis also provided information on the synthesis of PSII subunits and their assembly into complexes in both strains (Fig. 5*B*, autoradiograms). In wild-type cells the labeled D1 and D2 subunits were mainly found in RCCII(1) and RCCII(2), and their distribution between both complexes was approximately equal. Small amounts of labeled D1 and D2 (about 10% of the overall levels) were detected in the above-mentioned complexes RC* and RCa. However, these RC complexes accumulated only transiently, quickly disappeared during the chase (data not shown), and were not detected by immunoblot analysis. The inner

PSII antennae CP43 and CP47 were labeled less strongly and were present in the core complexes as well as in the fraction of unassembled proteins. In this fraction the labeling of CP43 was particularly strong. The labeling pattern in *gun4*⁻ thylakoids was different. The autoradiogram revealed that all four large PSII proteins in the assembled core complex RCCII(1) were labeled weakly and nearly to the same extent. The labeling of these proteins in RCCII(2) was hardly detectable. About 70% of the labeled D2, D1, and its partially processed form, iD1, were found in the RC* and RCa complexes. The remaining 30% were found in the core complexes (Fig. 5*B*, see Ref. 26). Moreover, the labeled proteins in RC* and RCa were detectable even after the 30 min of the radioactive chase (data not shown), showing that the complexes are not easily transformed into the core complexes. In the fraction of unassembled proteins, a strong labeling of CP43, but no labeling of CP47, was observed. The results confirmed that the insufficient synthesis of CP47 most probably limits the assembly and accumulation of the complete PSII core complexes. Similar transcript levels of the CP47-encoding gene *psbB* in wild type and *gun4*⁻ (not shown) showed

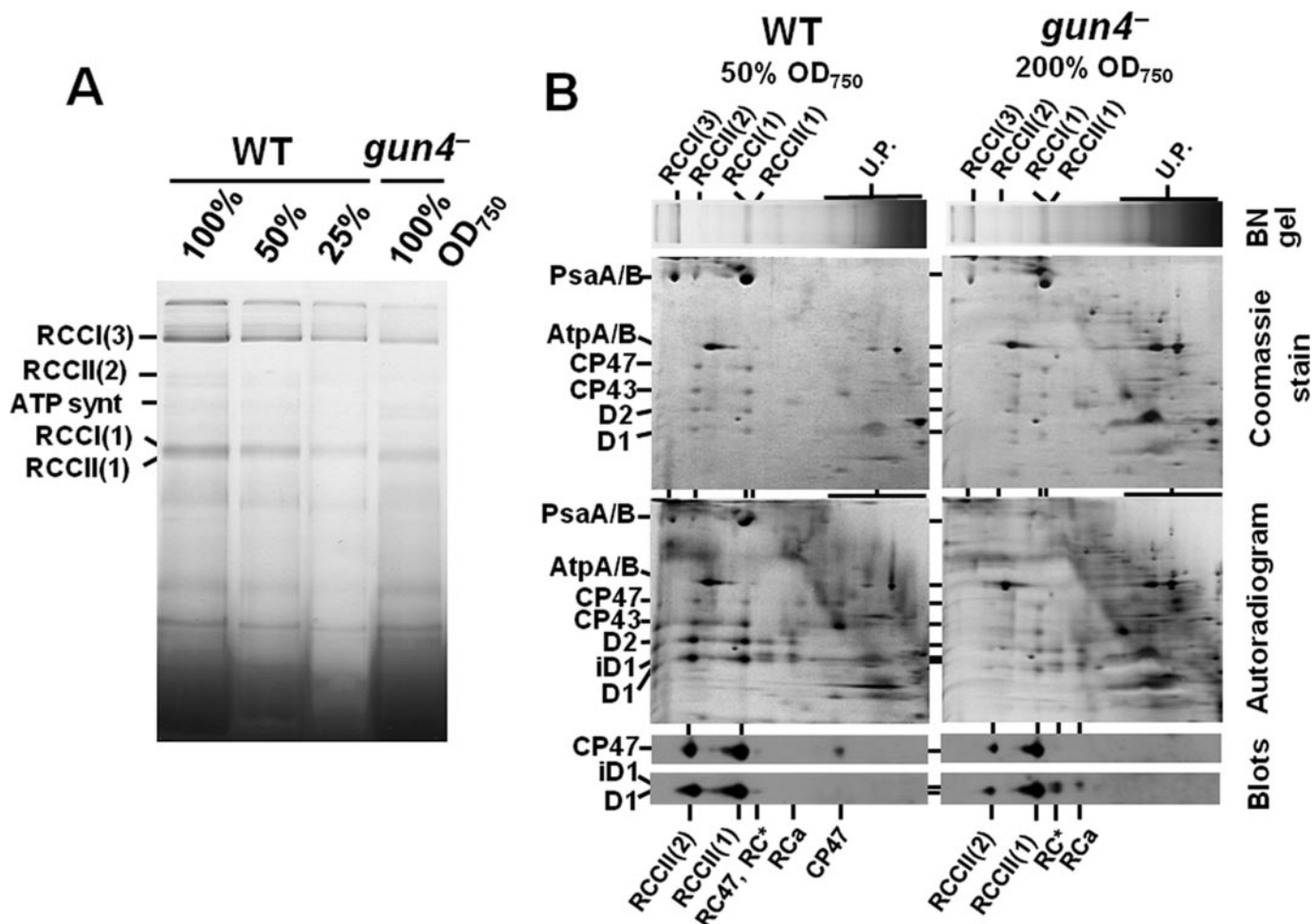


FIGURE 5. Analysis of membrane protein complexes and their assembly in wild-type and *gun4* mutant cells. A, membrane protein complexes from wild-type and *gun4* mutant labeled *in vivo* with ³⁵S]Met/Cys were separated in 4–16% BN gel as described under “Experimental Procedures.” Amounts of Chl corresponding to designated amounts of cells were loaded per each lane. B, two-dimensional analysis of membrane protein complexes separated in A. Equal amounts of Chl corresponding to the half (50% A₇₅₀) and double (200% A₇₅₀) amounts of wild-type and *gun4*⁻ cells were loaded, respectively. Lanes from the BN-gel were excised and placed on top of the 12–20% denaturing gel. The two-dimensional gels were either stained by Coomassie Blue (Coomassie gels), dried, and exposed to phosphorimaging plates (Autoradiograms) or electroblotted onto a polyvinylidene difluoride membrane that was immunodecorated using antibodies raised against D1 and CP47 proteins (blots). Designation of complexes: RCCI(3) and RCCI(1), trimeric and monomeric PSI complexes, respectively; RCCII(2) and RCCII(1), dimeric and monomeric PSII core complexes, respectively; RC47, the monomeric PSII core complex lacking CP43; RC* and RCa, reaction center complexes accumulating in PSII mutants lacking CP47 (24); U.P., unassembled proteins; iD1 designates D1 processing intermediate.

that the limitation in the accumulation of CP47 occurred at the posttranscriptional level.

DISCUSSION

In a previous work we did not succeed in preparing a fully segregated cyanobacterial *gun4* mutant in *Synechocystis* 6803. However, generation of such a mutant was feasible in a GT substrain of *Synechocystis* 6803. The *gun4* mutant was further examined for the function of Gun4 in tetrapyrrole biosynthesis and consecutive processes of protein assembly of the photosynthetic apparatus. Although *A. thaliana* GUN4 was reported to be nonessential for plant growth (10), here we show that the complete loss of *gun4* expression in a cyanobacterium prevents photoautotrophic growth. The Chl content in the *gun4* mutant grown at medium light intensities was dramatically reduced to about 8% that of the wild-type amount. At low light intensities the *gun4* mutant accumulated about 25% that of the wild-type Chl content. The better viability of the *gun4*⁻ cells under low light or

microaerobic conditions may be explained by the increased accumulation of P_{IX} in this mutant (Table 1) as P_{IX} is known to cause photooxidative damage (27). Recently, a very similar phenotype has been observed in a *Synechocystis* 6803 mutant that also accumulated large quantities of P_{IX}, resulting from a mutation in the ferrocyclase gene (28). However, at low light intensities under microaerobic conditions no further improvement in Chl accumulation was observed, with the *gun4* mutant accumulating about 25% of the wild-type Chl content. This suggests that the low light *gun4*⁻ phenotype can be mostly attributed to the lack of Chl precursors and not to enhanced photooxidative stress.

In agreement with the predicted function of Gun4 (10), the absence of this protein adversely affected the magnesium chelatase activity resulting in a limited supply of magnesium porphyrins for Chl synthesis (Table 1). In *Synechocystis* 6803, the Gun4 protein is present mainly as a soluble protein with a small fraction bound to the membrane (Fig. 3A). This is consistent with the situation described in *A. thaliana*, where the

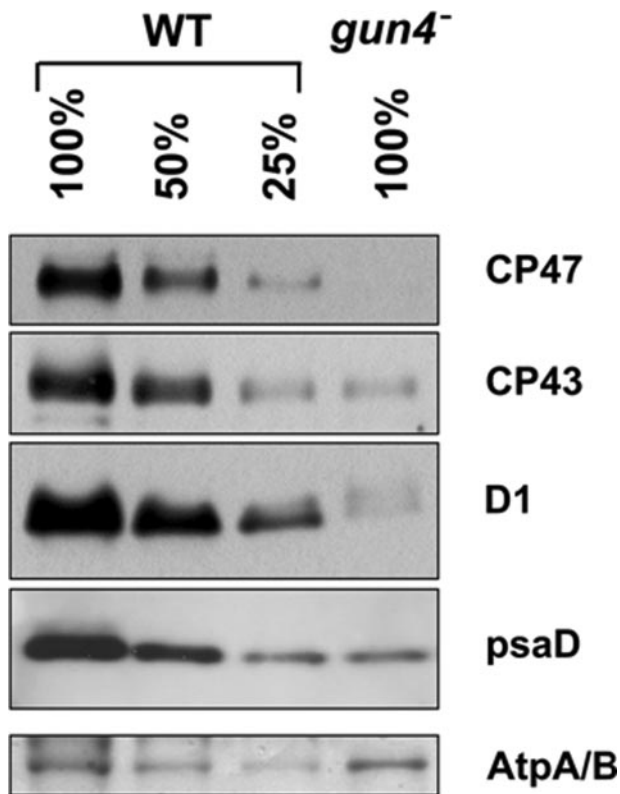


FIGURE 6. Semiquantitative immunoblot analysis of PSII and PSI subunits in wild type and *gun4* mutant. Wild-type and *gun4* mutant membrane fractions were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Different amounts of Chl corresponding to designated amounts of cells were loaded per each lane. Immunodetection was performed using polyclonal antibodies raised against the PSII subunits CP47, CP43, and D1, and the PSI subunit PsaD. ATPase band stained on the membrane with Ponceau red indicates protein loading.

majority of GUN4 was localized in the stroma and clearly smaller amounts in the envelope and thylakoid membranes (10). Moreover, the same authors reported that unlike the soluble GUN4, which probably is present as a monomer, the thylakoid-bound GUN4 was identified as part of a >500 kDa complex also containing the ChlH subunit (10). We have confirmed that cyanobacterial Gun4 is associated with ChlH as well (Fig. 4C) and showed that the accumulation of ChlH is influenced by absence of Gun4 (Fig. 3B). Interestingly, the proportion of the membrane-bound ChlH seemed to be decreased in the *gun4* mutant as well (Fig. 3A). Therefore, Gun4 is possibly important for association of ChlH with the membrane, maybe as a part of a membrane-bound magnesium chelatase complex.

The absence of the Gun4 protein also resulted in a decreased accumulation of ferrochelatase. Because no direct physical interaction between Gun4 and ferrochelatase was detected, the lack of Chl in *gun4*⁻ may influence the cellular content of ferrochelatase. The plant ferrochelatase as well as its cyanobacterial counterpart contains a highly conserved Chl binding motif within the C-terminal domain (2). Thus, we cannot exclude that a putative Chl binding to this domain is important for the synthesis or stability of ferrochelatase.

The *gun4* mutant provided a new opportunity to analyze the effect of Chl deficiency on photosynthetic membrane proteins in cyanobacterial cells grown under standard light conditions.

Previously, a comparably detailed study in the cyanobacterium *Plectonema boryanum* revealed that a mutation in *chlL*, which encodes a subunit of the light-independent protochlorophyllide oxidoreductase (29), leads to Chl deficiency during growth in nearly complete darkness. However, the consequences of Chl deficiency in the dark might significantly differ from those in the light, which is supported by the data of Kada *et al.* (29). The depletion of PSI Chl proteins was much faster than the depletion of PSII in the *chlL*⁻ strain. We assume that this difference is associated with the different stabilities of PSI and PSII Chl proteins in the dark and under illumination, respectively. Recently, it has been shown that the half-life of PSII-bound Chl in illuminated cells is remarkably shorter than that of the PSI-bound Chl (30). Within PSII, the turnover rate of unassembled CP43 is slower than that of other PSII Chl-binding proteins. In the absence of several PSII proteins, CP43 still accumulates to a larger extent (17). In the *gun4*⁻ mutant the cellular level of CP43 corresponded to the level of PSI proteins, both of which were reduced to about 25% that of the wild-type level. Moreover, the unassembled protein was clearly visible in the Coomassie-stained two-dimensional gel (Fig. 5B). Immunoblot analyses detected the incompletely processed form of D1, which is referred to as iD1, as a component of the RC complexes in the *gun4* mutant but not in wild-type cells (Fig. 5B, blot). The presence of the incompletely processed form of the D1 protein was reported to be closely related to Chl deficiency, underlying the hypothesis that maturation of D1 occurs after binding of Chl to the protein (31). Here we show that the incompletely processed form of D1 is present in the RC complexes and that the effect of Chl deficiency on the D1 maturation is indirect and related to the absence of CP47. Not until CP47 binds to the RC complexes can the complete and fast D1 maturation proceed.

Despite the presence of RC complexes, the subsequent PSII assembly intermediate RC47, which usually is formed from RC complexes after binding of CP47 (17), is absent in the *gun4* mutant. Because unassembled CP47 is not detectable in the mutant either (Fig. 5B, blot), it is clear that the accumulation of CP47 is strongly compromised in the mutant. A similar effect was detected in a CP47 mutant that harbors a few amino acid changes in the E-loop of the protein (32). This mutation resulted in a lowered cellular amount of functional PSII accompanied by an increased accumulation of RC complexes. Possibly, Chl deficiency limits the translation of CP47 to a greater extent than the translation of D1 due to the requirement of co-translational Chl binding to the CP47 apoprotein.

In conclusion, our results demonstrate that Gun4 plays an essential role in the magnesium branch of tetrapyrrole biosynthesis and dramatically affects the Chl content in the cell. 77 K fluorescence emission spectra as well as protein analyses demonstrated that Chl deficiency arising from the inactivation of *gun4* affects accumulation of both photosystems. The more extensive deficiency of PSII most probably was related to the insufficient accumulation of CP47. This Chl-binding protein appears to be the protein most sensitive to changes in the Chl availability and may, therefore, represent an important target for the mutual regulation of tetrapyrrole biosynthesis and formation of new PSII complexes.

Chlorophyll Deficiency in *Synechocystis gun4* Mutant

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