

Inactivation of the geranylgeranyl reductase (ChlP) gene in the cyanobacterium *Synechocystis* sp. PCC 6803

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Received 20 July 2004; received in revised form 3 November 2004; accepted 5 November 2004

Available online 18 November 2004

Abstract

Geranylgeranyl reductase catalyses the reduction of geranylgeranyl pyrophosphate to phytol pyrophosphate required for synthesis of chlorophylls, phylloquinone and tocopherols. The gene *chlP* (ORF *sl11091*) encoding the enzyme has been inactivated in the cyanobacterium *Synechocystis* sp. PCC 6803. The resulting $\Delta chlP$ mutant accumulates exclusively geranylgeranylated chlorophyll *a* instead of its phytolated analogue as well as low amounts of α -tocotrienol instead of α -tocopherol. Whereas the contents of chlorophyll and total carotenoids are decreased, abundance of phycobilisomes is increased in $\Delta chlP$ cells. The mutant assembles functional photosystems I and II as judged from 77 K fluorescence and electron transport measurements. However, the mutant is unable to grow photoautotrophically due to instability and rapid degradation of the photosystems in the absence of added glucose. We suggest that instability of the photosystems in $\Delta chlP$ is directly related to accumulation of geranylgeranylated chlorophyll *a*. Increased rigidity of the chlorophyll isoprenoid tail moiety due to three additional C=C bonds is the likely cause of photooxidative stress and reduced stability of photosynthetic pigment–protein complexes assembled with geranylgeranylated chlorophyll *a* in the $\Delta chlP$ mutant.

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Keywords: Chlorophyll biosynthesis; Geranylgeranyl reductase; Geranylgeranylated chlorophyll; *Synechocystis* sp. PCC 6803

1. Introduction

Chlorophyll (Chl) *a* plays a pivotal role in oxygenic photosynthetic organisms such as plants, green algae and cyanobacteria. Chl *a* is a primary electron donor in the

reaction centers (RCs) of photosystem (PS) I (P_{700}) and PSII (P_{680}). Moreover, Chl *a* constitutes also the primary electron acceptor (A_0) in RCI. A Chl *a* derivative—pheophytin *a*—is the primary electron acceptor in RCII. Most Chls in both PSI and PSII, however, act as light-harvesting (antenna) pigments, supplying the RCs with sufficient excitation energy to maintain a high rate of photosynthesis. In cyanobacteria, antenna Chl *a* is associated with the RC-core proteins: PsaA, PsaB of PSI and D1, D2, CP43, CP47 of PSII, respectively. Under certain stress conditions—in particular, iron deficiency—the Chl *a*-binding IsiA protein (CP43') accumulates in cyanobacteria; for a recent review, see Ref. [1]. IsiA was also found in a PSI *psaFJ*-null mutant of the cyanobacterium *Synechocystis* sp. PCC 6803 (in the following referred to as *Synechocystis*) under iron-replete conditions as a response to oxidative stress [2]. Additionally, one Chl *a* is present in the cytochrome *b₆f* complex

Abbreviations: Chl *a*, (phytylated) chlorophyll *a*; Chl *a_{gg}*, geranylgeranylated Chl *a*; Chlide *a*, chlorophyllide *a*; ChlG, chlorophyll synthase; ChlP, geranylgeranyl reductase; *chlP*, gene encoding ChlP; DCBQ, 2,6-dichloro-*p*-benzoquinone; GGPP, geranylgeranyl pyrophosphate; Km/Km^r, kanamycin/kanamycin resistance; OD, optical density; ORF, open reading frame; PBS, phycobilisome; PC, phycocyanin; PPP, phytol pyrophosphate; PSI, PSII, photosystem I, II; RC, reaction center; WT, wild type

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suggesting that this Chl *a* plays a structural role in assembly and stabilization of the complex [3]. Chl *a* availability affects accumulation of Chl *a*-binding apoproteins of PSI and PSII in higher plants and cyanobacteria [4–6]. Conceivably, the isoprenoid tail moiety of Chl *a* appears to be of particular importance: its absence was shown to prevent, e.g., accumulation of D1 and PsaA [6].

Both the tetrapyrrole and the isoprenoid synthesizing pathways provide the substrates for the final steps of Chl biosynthesis, chlorophyllide (Chlide) and geranylgeranylpyrophosphate (GGPP) (Fig. 1). In the last step of Chl *a* biosynthesis, Chlide is esterified with GGPP or phytol pyrophosphate (PPP) resulting in formation of geranylgeranylated Chl *a* (Chl *a*_{gg}) or phytylated Chl *a*, respectively (for reviews, see Refs. [7,8]). The reaction is catalyzed by Chl synthase (ChlG) (Fig. 1). GGPP has four C=C (double) bonds, three of which (at positions 6, 10, and 14) are reduced by geranylgeranyl reductase (ChlP). ChlP accepts the substrates GGPP as well as Chl *a*_{gg} yielding PPP or Chl *a*, respectively (Fig. 1, cp. also Ref. [9]).

Phytol is also the lipophilic tail moiety of phyloquinone (vitamin K₁) and tocopherols (vitamin E). Phyloquinone acts as secondary electron acceptor (A₁) in RCI [10]. Tocopherols are lipophilic antioxidants being synthesized only in photosynthetic organisms [11,12].

Naturally, Chl *a*_{gg} occurs in detectable quantities only transiently in etioplasts of higher plants during greening [13,14]. It is generally not detectable in the mature photosynthetic apparatus of plants, green algae and cyanobacteria. The photosynthetic purple bacterium, *Rhodospirillum rubrum*, accumulates geranylgeranylated bacteriochlorophyll *a* (BChl *a*_{gg}). The RC of this species, however, contains phytylated bacteriopheophytin at the H site [15]. Previously characterized mutants of the green alga *Scenedesmus obliquus*

and the purple bacterium *Rhodobacter capsulatus* accumulating Chl *a*_{gg} and BChl *a*_{gg}, respectively, were shown to be impaired in photosynthetic performance [16,17].

Transgenic *Nicotiana tabacum* lines expressing antisense *chlP*-RNA showed reduced Chl and tocopherol contents and accumulated considerable levels of Chl *a*_{gg}, Chl *b*_{gg} and α -tocotrienol in their leaves [18–20]. These transgenic plants, however, did not completely substitute Chls *a* and *b* by geranylgeranylated counterparts. Nevertheless, increased light sensitivity and impaired growth were observed [18–20]. The specific roles of tocopherols in protection of thylakoid membranes from photooxidation and stabilization of the photosynthetic apparatus remain still elusive. The effects brought about by substitution of Chl *a* by Chl *a*_{gg}, however, indicate pivotal functions of the phytol tail moiety in photosynthesis that cannot be substituted for completely by a geranylgeranyl residue.

The present study is aimed at contributing to a better understanding of the role of the isoprenoid chain, in particular the phytol chain of Chl *a*, in the structure and function of the photosynthetic apparatus. The *chlP* gene (ORF *sll1091*) encoding geranylgeranyl reductase (ChlP) [21] was inactivated in *Synechocystis*. The resulting Δ *chlP* mutant was used to study the effects of the exclusive accumulation of Chl *a*_{gg} (instead of its phytylated analogue) on assembly, stability and function of the photosynthetic apparatus.

2. Materials and methods

2.1. Strains and growth conditions

Synechocystis wild-type (WT) and Δ *chlP* mutant cells were grown in BG11 medium [22] supplemented with 10

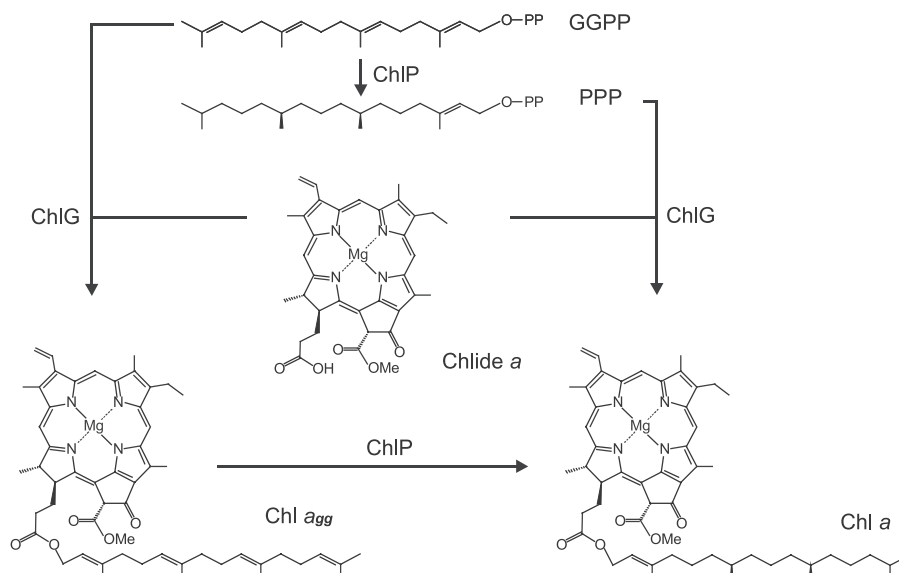


Fig. 1. The last steps of chlorophyll *a* biosynthesis. GGPP, geranylgeranyl pyrophosphate; PPP, phytol pyrophosphate; Chl *a*_{gg}, geranylgeranylated Chl *a*; Chl *a*, phytylated Chl *a*; ChlP, geranylgeranyl reductase; ChlG, Chl synthase.

mM glucose at 40 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (moderate light intensity) unless otherwise indicated. The $\Delta chlP$ mutant was propagated at low light intensity (2–4 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). For growth on plates, 1.0% (w/v) bacto agar (Difco) was added to BG11. Additionally, the medium was supplemented with kanamycin (Km, 50 $\mu\text{g ml}^{-1}$) in the case of the $\Delta chlP$ mutant. Growth was monitored by optical density (OD) measurements at 750 nm using an Ultrospec II spectrophotometer (LKB Biochrom).

2.2. Mutant construction

A DNA fragment carrying a segment of the *chlP* coding region was amplified by PCR from genomic DNA of the *Synechocystis* WT strain using the primers CGGGAA CGGGCGGAGAACTGGG (F) and CACATACGGGCGGATTGGCGGC (R) (Fig. 2A). The PCR product was cloned into the pGEM-T Easy vector (Promega, Mannheim, Germany). A Km resistance (Km^r) cassette was cut with *HincII* from the pUC4K plasmid (Pharmacia) and inserted into the *Eco72I* site of the cloned *chlP* fragment. For cloning and subcloning of plasmids, *Escherichia coli* strain JM109 [23] was used. The resulting plasmid construct was used to transform *Synechocystis* WT cells to Km^r by homologous recombination. Transformants, in which the Km^r cassette interrupts the WT gene (cp. Fig. 2A), were segregated by restreaking on plates containing 10 mM glucose and increasing concentrations of Km (from 20 to 100 $\mu\text{g ml}^{-1}$). Low light conditions were applied during segregation to avoid generation of suppressor mutations. Complete segregation of the *Synechocystis* mutant was

confirmed by PCR (Fig 2B). The homozygous mutant with a disrupted *chlP*-gene has been designated $\Delta chlP$.

2.3. Pigment and tocopherol analyses

Pigments were extracted from whole cells with 90% methanol. Concentrations of Chls and carotenoids were determined according to Ref. [24]. Pigments were separated by HPLC on a Waters chromatography system (600 E gradient module, 717 autosampler, 996 diode array detector) using a Waters Spherisorb ODS2 Analytical Column (5- μm particle size; 4.6 $\times$ 250 mm). Gradients consisted of acetonitrile/water/triethyl amine (900:100:1, solvent A) and ethyl acetate (solvent B) with 100% A for 16 min, 66.7% A/33.3% B for 6 min, 59.7% A/40.3% B for 12 min, 33.3% A/66.7% B for 0.2 min, 100% B for 2.5 min, and 100% A for 4.3 min. The flow rate was 1 ml min^{-1} . Pigments were identified by their specific retention times and absorption spectra and quantified by comparison with authentic standards using photodiode-array detection at 440 nm.

Tocopherols were extracted from whole cells with 90% methanol and separated by HPLC on a Nova Park C_{18} column (4- μm particle size; 3.9 $\times$ 150 mm) at 1 ml min^{-1} with the following gradient: 30% methanol and 10% 0.1 M ammonium acetate, pH 5.2 (solvent A) and 100% methanol (solvent B): 6% A/94% B for 7 min, 1% A/99% B for 13 min, and 6% A/94% B for 5 min. Tocopherols were identified by fluorescence detection (λ_{ex} =295 nm, λ_{em} =325 nm) and comparison to authentic standards (Roth, Germany).

2.4. Absorption and 77 K fluorescence emission spectra

Absorption spectra of whole cells in BG11 medium were recorded on an Ultrospec II spectrophotometer. 77 K fluorescence emission spectra were obtained using a Fluorolog FL-112 spectrofluorimeter with a model 1680 emission double monochromator (Jobin-Yvon, Longjumeau, France). Cells were resuspended in 25 mM HEPES–NaOH, pH 7.0 containing 67% glycerol to an OD=1.0 at 750 nm corresponding to Chl *a* concentrations of 2.6 and 1.9 $\mu\text{g ml}^{-1}$, for WT and the $\Delta chlP$ mutant, respectively, for excitation at 435 nm (Chl *a*). In experiments with phycobilisome (PBS) excitation (at 590 nm) glycerol was omitted.

2.5. Oxygen evolution measurements

Photosynthetic activity was measured as oxygen evolution from intact cells at 30 °C under saturating white light (3000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) using a Clark-type electrode essentially as described [25]. Cells were resuspended in BG11 medium buffered with 25 mM HEPES–NaOH, pH 7.0 containing 10 mM glucose to an OD=1.5 at 750 nm. Whole-chain electron transport rates were measured in the presence of 10 mM NaHCO_3 . PSII-mediated electron transport was measured in the presence of 10 mM NaHCO_3 ,

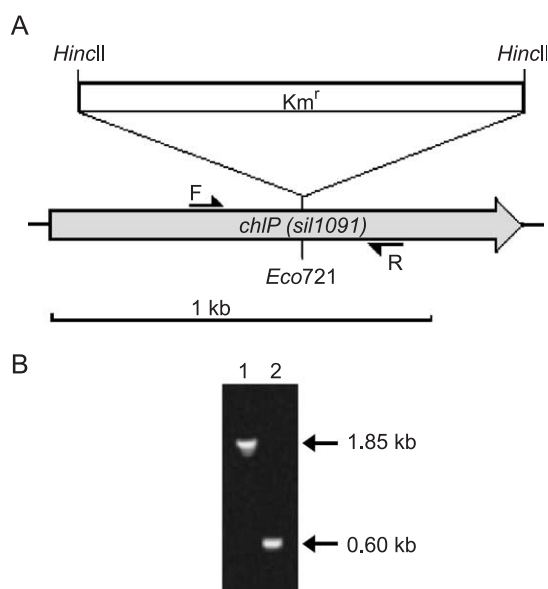


Fig. 2. Insertional inactivation of the *chlP* gene in *Synechocystis* sp. PCC 6803. (A) Scheme of the insertion of the kanamycin resistance (Km^r) cassette into the *Eco72I* site of the *chlP* gene. Arrows indicate the positions of the forward (F) and reverse (R) primers used for genomic PCR. (B) PCR products amplified from the $\Delta chlP$ mutant (lane 1) and WT (lane 2).

1 mM $K_3Fe(CN)_6$ and varying concentrations of 2,6-dichloro-*p*-benzoquinone (DCBQ).

3. Results and discussion

3.1. Mutant construction and growth characteristics

A segment of the *chlP* coding region was amplified by PCR from *Synechocystis* WT genomic DNA (Fig. 2A). A Km^r cassette was inserted into the *Eco72I* site of the cloned *chlP* fragment. The resulting plasmid construct was used to transform WT cells to Km^r through homologous recombination. Segregation of the transformants was achieved by adding increasing concentrations of Km (from 20 to 100 $\mu g\ ml^{-1}$) and verified by PCR (Fig. 2B) yielding the homozygous $\Delta chlP$ mutant strain.

The $\Delta chlP$ mutant is incapable of photoautotrophic growth (Table 1). In the presence of 10 mM glucose in the medium (photomixotrophic conditions), the doubling time of $\Delta chlP$ is approximately equal to that of WT under 2–4 or 40 $\mu mol\ photons\ m^{-2}\ s^{-1}$ illumination (Table 1). However, the mutant did not grow at light intensities above 100 $\mu mol\ photons\ m^{-2}\ s^{-1}$, not even when it was supplemented with glucose.

3.2. Pigment and tocopherol contents

Cultures of the $\Delta chlP$ mutant grown under moderate or low light conditions were more bluish in color than the WT. The difference in coloration is corroborated by whole cell absorption spectra (Fig. 3). Whereas peaks at 440 and 680 nm (corresponding to Chl) are reduced in $\Delta chlP$, a peak centered at 625 nm corresponding to phycocyanin (PC)—the major component of phycobilisomes (PBS)—is increased (Fig. 3).

Spectrophotometric quantitation of pigments following extraction with methanol consistently revealed a 26% decrease of Chl content and a 22% decrease of total carotenoid content in the mutant as compared to WT (Table 1). With the exception of a slight relative increase in myxoxanthophyll,

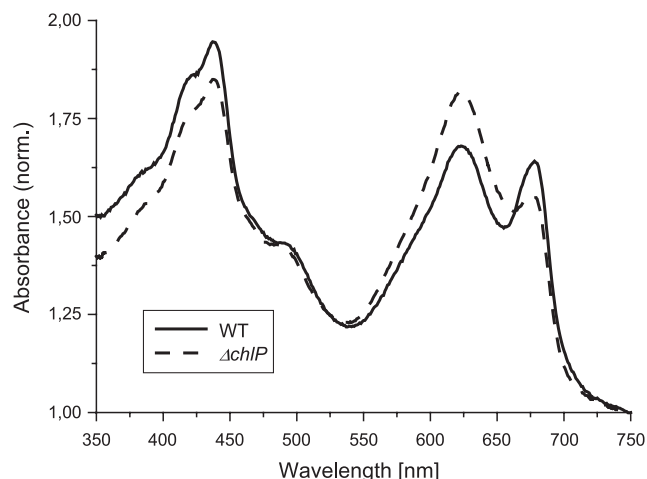


Fig. 3. Absorption spectra of whole cells of *Synechocystis* sp. PCC 6803 WT (solid line) and $\Delta chlP$ mutant (dashed line). Spectra were normalized to 1.0 at 750 nm. This procedure facilitates comparison on a per cell basis.

carotenoid composition was not significantly altered in $\Delta chlP$ (Fig. 4). HPLC analyses indicated that exclusively Chl a_{gg} accumulates in $\Delta chlP$ mutant cells (Fig. 4B).

A possible contribution of Chlide *a* to Chl absorption in the mutant was assessed after removal of Chl a_{gg} with *n*-hexane. No Chlide was detectable in the mutant extracts by spectrophotometry. Thus, the 440- and 680-nm peaks in the $\Delta chlP$ absorption spectrum indeed entirely correspond to Chl a_{gg} .

The contents of tocopherols were also determined in WT and $\Delta chlP$ mutant. Whereas the WT accumulates exclusively α -tocopherol, $\Delta chlP$ contains only α -tocotrienol instead (Fig. 5). The amount of tocotrienol in the mutant, however, does not reach the WT vitamin E contents, suggesting less efficient prenylation of homogentisate with GGPP by the respective prenyltransferase, consistent with *in vitro* experiments [26].

Table 1

Growth rates and pigment composition in *Synechocystis* sp. PCC 6803 WT and $\Delta chlP$ mutant cells

	WT	$\Delta chlP$
Photoautotrophic growth, doubling time [h]		
2–4 $\mu mol\ photons\ m^{-2}\ s^{-1}$	47.1 $\pm$ 3.1	No growth
40 $\mu mol\ photons\ m^{-2}\ s^{-1}$	11.2 $\pm$ 1.1	No growth
Photomixotrophic growth, doubling time [h]		
2–4 $\mu mol\ photons\ m^{-2}\ s^{-1}$	17.3 $\pm$ 1.8	16.0 $\pm$ 1.4
40 $\mu mol\ photons\ m^{-2}\ s^{-1}$	9.4 $\pm$ 0.9	9.2 $\pm$ 0.7
100 $\mu mol\ photons\ m^{-2}\ s^{-1}$	8.8 $\pm$ 0.6	No growth
Chlorophyll content [$\mu g\ ml^{-1}\ OD_{750}^{-1}$] ^a	2.61 $\pm$ 0.27	1.93 $\pm$ 0.18
Total carotenoid content [$\mu g\ ml^{-1}\ OD_{750}^{-1}$] ^a	0.69 $\pm$ 0.06	0.54 $\pm$ 0.04

^a Pigments were extracted from cells grown photomixotrophically at a light intensity of 40 $\mu mol\ photons\ m^{-2}\ s^{-1}$.

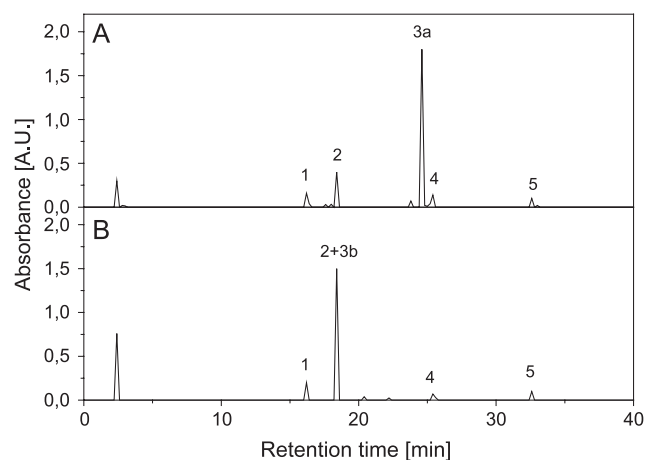


Fig. 4. HPLC analysis of pigment extracts from WT and $\Delta chlP$ cells of *Synechocystis* sp. PCC 6803. Absorbance was measured at 440 nm. Peaks: 1, myxoxanthophyll; 2, zeaxanthin; 3a, phytylated chlorophyll *a*; 3b, geranylgeranylated chlorophyll *a*; 4, echinenone; 5, β -carotene.

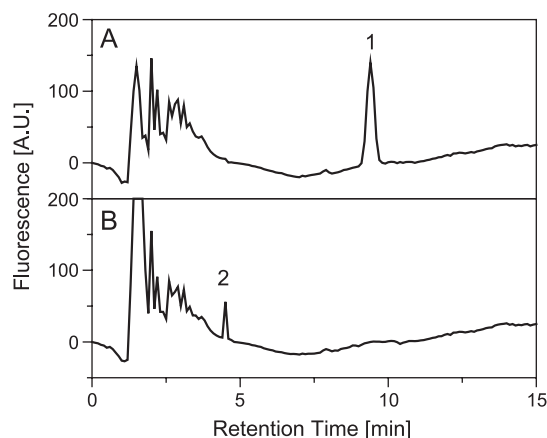


Fig. 5. HPLC analysis of vitamin E contents in WT and $\Delta chlP$ cells of *Synechocystis* sp. PCC 6803. Fluorescence was excited at 295 nm and measured at 325 nm. Peaks: 1, α -tocopherol; 2, α -tocotrienol.

3.3. 77 K fluorescence emission spectra

Consequences of the substitution of Chl *a* by Chl a_{gg} on assembly, stoichiometry, and structure of PSI and PSII were assessed using 77 K fluorescence emission spectroscopy. Excitation of Chl at 435 nm elicits fluorescence emission with prominent maxima at 685, 695 and 725 nm in WT (Fig. 6A). The former two maxima represent emission from the PSII core antenna complexes, CP43 and CP47, respectively. A certain proportion of the 685-nm peak has to be assigned also to the long-wavelength terminal emitter of PBS [27]. It has been shown previously that accumulation of the IsiA protein (CP43') as a result of iron deficiency and/or photooxidative stress may cause an increase in the 685-nm emission, too [1,2]. The dominant emission peak at 725 nm derives from PSI [2,25,27,28]. Minor peaks at about 650 and 665 nm originate from the

PBS components, PC and allophycocyanin, respectively [29].

The 77 K fluorescence spectrum of the $\Delta chlP$ mutant is characterized by a considerable reduction of the PSI peak being consistent with the decreased cellular Chl content. Moreover, the maximum of this peak in the mutant is blue-shifted by 2 nm as compared to WT, possibly indicating a somehow perturbed association of Chls a_{gg} with the respective apoprotein(s) affecting the structure of PSI in the $\Delta chlP$ mutant. A slight blue-shift of PSI emission has also been observed upon accumulation of the IsiA protein in *Synechocystis* [2]. Additionally, the mutant spectrum reveals an enhanced fluorescence at 685 nm. The latter might be due to an altered CP43-to-CP47 ratio, or to compromised excitation energy transfer from CP43 to CP47. Another explanation might be that the enhanced 685-nm peak in the $\Delta chlP$ fluorescence spectrum is due to increased emission from the terminal emitter of the PBS. This is conceivable since the amount of PBS is enlarged in mutant cells as judged from the absorption spectra (cp. Fig. 3). The notion is supported by the 77 K fluorescence emission spectra obtained upon excitation at 590 nm (the short-wavelength edge of the PBS absorption band, Fig. 6B). Glycerol was not added to the samples to avoid decoupling of PBS from the photosystems. The WT spectrum obtained upon 590-nm excitation exhibits four maxima corresponding to emission from PC (at about 655 nm), allophycocyanin (shoulder at about 665 nm), the terminal emitter/CP43/IsiA (at 685 nm), and PSI (at 725 nm) (Fig. 6B, cp. also Refs. [2,27–29]). The respective spectrum of the $\Delta chlP$ mutant reveals a dramatic increase of the 685-nm peak. The estimated ratio of the 685-nm peak to the 665-nm peak is approximately 9. This value is consistent with 685 nm-to-665 nm fluorescence ratios of 6–10 as observed for isolated PBS [30]. Hence, the data suggest that also the increase of the 685-nm peak in the

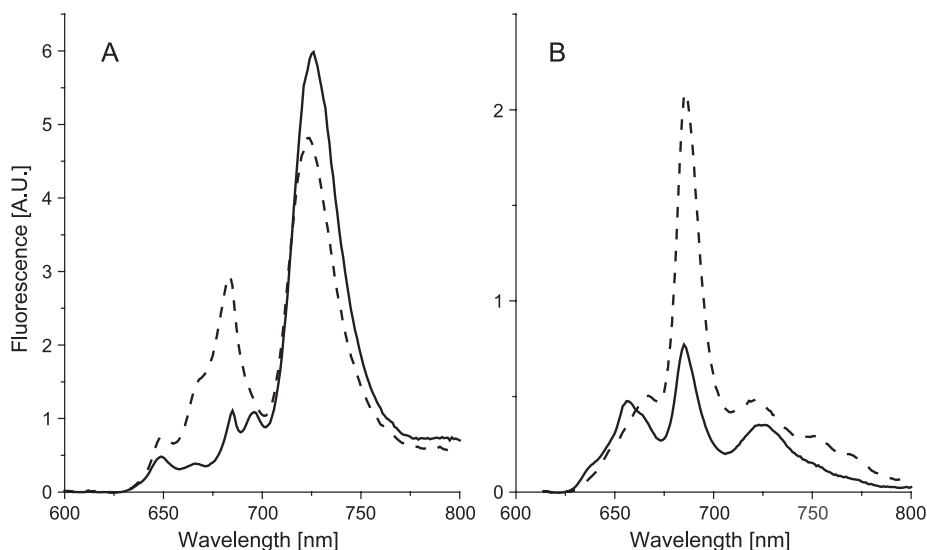


Fig. 6. 77 K fluorescence emission spectra of whole cells of *Synechocystis* sp. PCC 6803 WT (solid line) and $\Delta chlP$ mutant (dashed line). Cells were grown photomixotrophically under moderate ($40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) light conditions. Cells were suspended in 67% glycerol/25 mM HEPES–NaOH (pH 7.0) for excitation at 435 nm (A). Glycerol was omitted for excitation at 590 nm (B).

AchlP spectrum recorded upon Chl excitation is likely due to the PBS terminal emitter. Possibly, a proportion of the PBS is not tightly coupled energetically to the photosystems in the *AchlP* mutant. Alternatively, at least a fraction of the increased 685-nm emission peak may derive from the IsiA (CP43') protein which has been shown to accumulate in cyanobacteria in response to a variety of stress conditions [1,2]. The latter would also be consistent with the observed blue-shift of the main PSI emission maximum [2].

3.4. Electron transport

The 77 K fluorescence emission spectra obtained upon Chl excitation indicate the presence of PSI and PSII in *AchlP* cells. Functional characterization of PSI and PSII was substantiated by measuring electron transport as oxygen evolution under light-saturated conditions. The rate of electron transport across the whole chain (from H₂O to CO₂) appeared to be increased by 34% in *AchlP* cells in comparison to WT, indicating the presence of fully functional PSI and PSII in the mutant (Table 2).

PSII-mediated electron transport was measured in the presence of the artificial electron acceptors, DCBQ and 1 mM K₃Fe(CN)₆. In WT cells the 0.5 mM DCBQ-mediated oxygen evolution rate was about 38% higher than the whole chain rate. In contrast, under the same conditions *AchlP* evolved oxygen at an essentially lower rate, even less than the corresponding whole chain rate (Table 2).

The latter observation suggests an inhibitory effect of DCBQ on PSII in the mutant. Therefore, oxygen evolution was determined in mutant and WT cells in dependence on increasing concentrations of DCBQ (from 0.2 to 1.0 mM). Maximum oxygen evolution was achieved in WT at 0.5 mM DCBQ (Table 2). In contrast, increasing DCBQ concentrations were accompanied by a significant decline in oxygen evolution in the *AchlP* mutant (Table 2). At 1.0 mM DCBQ oxygen evolution of mutant cells was only about 20% of the whole chain rate. The origin of the inhibitory effect of DCBQ is presently unclear and requires further study. One possible explanation might be that quinone electron acceptors (such as DCBQ) can act as nonphotochemical quenchers of Chl excited states—in particular in structurally altered PSII antenna systems [31],

thus reducing excitation density available for photochemistry in the *AchlP* mutant.

In addition to increased electron transport capacity, the mutant shows enhanced respiration as judged by an elevated oxygen uptake rate in the dark when supplemented with glucose (data not shown). Considering the similarity of the *AchlP* and WT growth rates under moderate light conditions, these data suggest the existence of additional energy-consuming processes in the mutant.

3.5. Instability of the photosystems in the *AchlP* mutant

A previously described mutant of *R. capsulatus* accumulating BChl *a*_{gg} (instead of BChl *a*) showed compromised photosynthetic performance due to instability of components of the photosynthetic apparatus [17]. A similar impact can also be asserted for Chl *a*_{gg} accumulation in the *AchlP* mutant. To test this hypothesis, *AchlP* mutant and WT cultures were depleted of exogenous glucose supply. Cells grown photomixotrophically were gently harvested by centrifugation and transferred to a glucose-free medium. Growth of the cultures was assessed as OD at 750 nm. The cellular PS content was monitored by absorption and 77 K fluorescence spectroscopy (not shown) and parallel measurements of Chl content (Fig. 7B). To account for a possible additional impact of illumination, Chl degradation was compared in light and in darkness.

Following transfer to photoautotrophic conditions, the *AchlP* mutant continued growth for only 1 day (Fig. 7A). Thereafter growth ceased completely in the mutant, in contrast to WT. Both strains, *AchlP* and WT, exhibited no growth in the dark. The Chl content (Fig. 7B) of the dark-incubated WT did not show significant alterations, indicating stability of PSI and PSII. In contrast, a continuous decline of Chl content was observed in *AchlP* cultures during dark incubation. This observation can be explained with progressive degradation of the mutant's photosystems (Fig. 7B). Light was found to promote the degradation process in *AchlP* (Fig. 7B). The latter observation is corroborated by a rapid Chl depletion observed in glucose-supplemented mutant cultures under enhanced illumination (500 μmol photons m⁻² s⁻¹; data not shown).

The observed instability of the photosystems in the *AchlP* mutant is most likely due to impaired binding of Chl *a*_{gg} to the respective apoproteins due to reduced flexibility of the isoprenoid chain. Three additional C=C bonds will hinder rotation of carbon atoms at six positions, thereby limiting the number of possible conformations of the isoprenoid tail. It has been shown previously that the phytyl tail moiety was required for the stable assembly of the D1 and PsaA complexes [6]. Additionally, perturbed binding of Chl *a*_{gg} to the apoproteins—in particular of accessory light-harvesting pigments—may lead to impairment of excitation energy transfer to the RCs and, hence, to longer excited state life-times, inevitably giving rise to enhanced generation of long-lived Chl triplet excited states.

Table 2
Electron transport rates in *Synechocystis* sp. PCC 6803 WT and *AchlP* mutant cells

Oxygen evolution rates [μmol O ₂ h ⁻¹ OD ₇₅₀ ⁻¹]	WT	<i>AchlP</i>
Whole chain ^a	576 ^b	774
0.2 mM DCBQ ^c	778	463
0.5 mM DCBQ	795	293
0.8 mM DCBQ	694	174
1.0 mM DCBQ	645	146

^a In the presence of 10 mM NaHCO₃.

^b S.D. were about ±10%.

^c In the presence of 10 mM NaHCO₃, DCBQ and 1.0 mM K₃Fe(CN)₆.

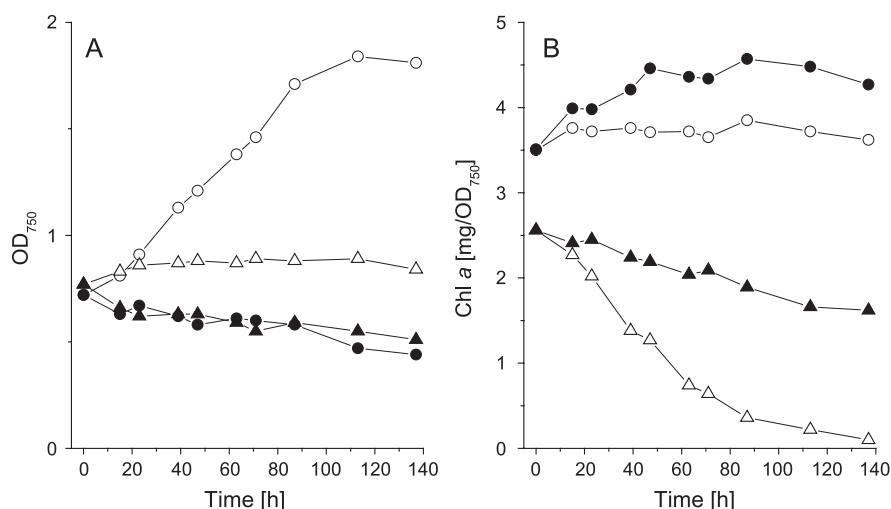


Fig. 7. Growth (A) and Chl content (B) in cells of *Synechocystis* sp. PCC 6803 WT (circles) and *AchlP* mutant (triangles). Cultures were cultivated under moderate ($40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) light conditions (open symbols) or in the dark (full symbols) in the absence of glucose.

The latter are known to react readily with oxygen ultimately leading to photodamage of pigment–protein complexes and photosynthetic membranes. Accumulation of the IsiA protein (as suggested by the increased 685-nm fluorescence and the shift of the PSI emission peak) may be indicative of photooxidative stress in the mutant [1,2]. Correct assembly of Chls with the apoprotein may also be essential for efficient photoprotection (by carotenoids and/or other antioxidants). Alterations in antioxidant (e.g., vitamin E) contents—although most probably not being the primary cause—may further exacerbate the observed instability of the photosystems (see also below).

An enhanced rate of photosystem turnover in the mutant cells is a likely consequence of their instability. Glucose requirement and more intense respiration are indicative for intensive metabolic activity that is required to maintain the observed steady-state levels of otherwise degraded photosystems in the *AchlP* mutant.

3.6. Complementation

To exclude contributions of secondary mutations to the *AchlP* phenotype, the WT *chlP* copy was reintroduced into the mutant. The mutant was transformed with a 0.60-kb *chlP* segment to oust the Km^r cassette by homologous recombination and restore the WT *chlP* gene activity. The resultant transformants were selected and restreaked on plates under standard photoautotrophic conditions. After a month of photoautotrophic propagation transformants had lost the resistance to Km and complete segregation of the WT *chlP* copy was confirmed by PCR (data not shown). The transformants regained the ability to synthesize phytylated Chl *a* and tocopherols and exhibited color, absorption spectra and oxygen evolution rates characteristic of WT (data not shown). Hence, the phenotype of the *AchlP* mutant is entirely caused by inactivation of the *chlP* gene.

It should be noted that attempts to obtain spontaneous suppressor mutations restoring photoautotrophic capability were unsuccessful so far. The latter indicates that (i) the adverse effects of Chl *a*_{gg} accumulation on photosynthetic growth cannot be completely compensated by any other physiological mechanism; and (ii) in *Synechocystis* ChlP function is coded for exclusively by ORF *slI1091*.

4. Concluding remarks

The current study was conducted to investigate the impact of substitution of (phytylated) Chl *a* by its geranygeranylated counterpart on assembly, stability and performance of the photosynthetic apparatus. ChlP activity, which provides the phytyl moiety for Chl (as well as for phylloquinone and tocopherol) synthesis, is exclusively encoded by ORF *slI1091* in *Synechocystis*. To discern the effects of Chl *a*_{gg} accumulation and tocopherol or phylloquinone deficiency, the *AchlP* strain was also compared to respective deficient mutants. In our laboratories tocopherol and phylloquinone deficient mutants with disrupted homogenisate phytyl transferase (*hpt*, ORF *slr1736*) and 1,4-dihydroxy-2-naphthoic acid phytyl transferase (*menA*, ORF *slr1518*) genes, respectively, have been constructed, too. These mutants show phenotypes similar to previously described analogous *Synechocystis* mutants [10,26,32]. Tocopherols are apparently not essential for photosynthesis and photoautotrophic growth in *Synechocystis* (Ref. [26] and own unpublished results). Even tolerance to high light is not affected in tocopherol-deficient *Synechocystis* mutants [26]. Consequently, *AchlP* has to be compared in particular to the phylloquinone-deficient mutant *ΔmenA*. Two phenotypic similarities were observed between *AchlP* and *ΔmenA*: (i) a reduced cellular Chl content consistent with a decreased PSI level; and (ii) inability to grow under illumination exceeding $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ [10].

However, differences do also exist: in contrast to *ΔchlP*, the *ΔmenA* mutant grows photoautotrophically under moderate illumination [10]. Whereas photomixotrophic growth (under moderate light) is not affected in *ΔchlP*, the *ΔmenA* mutant exhibited about twofold increased doubling times as compared to WT under the same conditions [10]. Contrasting Chl a_{gg} and phyloquinone-related contributions to the *ΔchlP* phenotype requires further investigations—in particular regarding individual electron transfer steps in which phytylated electron carriers (in WT) are substituted by geranylgeranylated ones (in the *ΔchlP* mutant). These intricate investigations are currently under way in our labs. In this regard, it is worth to note that overall linear electron transport appears not to be affected directly by the *ΔchlP* mutation, although several components of the electron transport chain in both PSI and PSII are natively phytylated in WT.

The observed enlarged amount of PBS might be a compensatory response to account for impaired light-harvesting (excitation energy transfer) capacity of Chl a_{gg} containing antenna complexes in the *ΔchlP* mutant (see above). However, the instability of certain Chl-binding proteins in the *ΔchlP* mutant apparently compromises coupling of PBS to the photosystems.

Very recently, rice mutants with alterations in hydrogenation of the Chl alcohol side chain have been isolated [33]. One of these mutants showed preferential accumulation of Chls a_{gg} and b_{gg} . The mutant was lethal despite a high photosynthetic activity measured as oxygen evolution. These authors concluded that accumulation of geranylgeranylated Chls leads to impaired photoprotection as well as instability of the photosystems [33].

In conclusion, the *ΔchlP* mutant is a useful system for further studies of the aspects of Chl–apoprotein interactions, photosystem biogenesis and function as well as regulation of photosynthetic and respiratory activities.

Acknowledgements

The authors thank Barbara Hickel for assistance with HPLC analyses, Dr. Roman Lebedev and Juri Fantin for help with oxygen evolution measurements as well as Dr. Annegret Wilde and Victor Melnik for helpful advice. The work was supported by the Deutsche Forschungsgemeinschaft (SFB 429, TPs A2 and B9) and grants from the Russian Foundation of Basic Research (N 04-04-48200) and the Russian Program of Leading Scientific Institutions.

References

- [1] K.-P. Michel, E.K. Pistorius, Adaptation of the photosynthetic electron transport chain in cyanobacteria to iron deficiency: the function of IdIA and IsiA, *Physiol. Plant.* 120 (2004) 36–50.
- [2] R. Jeanjean, E. Zuther, N. Yermenko, M. Havaux, H.C.P. Matthijs, M. Hagemann, A photosystem 1 *psaFJ-null* mutant of the cyanobacterium *Synechocystis* PCC 6803 expresses the isiAB operon under iron replete conditions, *FEBS Lett.* 549 (2003) 52–56.
- [3] G. Kurisu, H. Zhang, J.L. Smith, W.A. Cramer, Structure of the cytochrome b_6f complex of oxygenic photosynthesis: tuning the cavity, *Science* 302 (2003) 1009–1014.
- [4] L.A. Eichacker, J. Soll, P. Lauterbach, W. Rüdiger, R.R. Klein, J.E. Mullet, In vitro synthesis of chlorophyll *a* in the dark triggers accumulation of chlorophyll *a* apoproteins in barley etioplasts, *J. Biol. Chem.* 265 (1990) 13566–13571.
- [5] Q.F. He, W. Vermaas, Chlorophyll *a* availability affects *psbA* translation and D1 precursor processing in vivo in *Synechocystis* sp. PCC 6803, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 5830–5835.
- [6] J. Kim, L.A. Eichacker, W. Rüdiger, J. Mullet, Chlorophyll regulates accumulation of the plastid-encoded chlorophyll proteins P700 and D1 by increasing apoprotein stability, *Plant Physiol.* 104 (1994) 907–916.
- [7] S. Beale, Biosynthesis of cyanobacterial tetrapyrrole pigments: hems, chlorophylls and phycobilins, in: D.A. Bryant (Ed.), *The Molecular Biology of Cyanobacteria*, Kluwer Academic Publishers, Dordrecht, 1993, pp. 519–558.
- [8] W. Rüdiger, Chlorophyll metabolism: from outer space down to molecular level, *Phytochemistry* 46 (1997) 1151–1167.
- [9] W. Rüdiger, S. Schoch, The last steps of chlorophyll biosynthesis, in: H. Scheer (Ed.), *Chlorophylls*, CRC Press, Boca Raton, 1991, pp. 451–464.
- [10] T.W. Johnson, G. Shen, B. Zybailov, D. Kolling, R. Reategui, S. Beauparlant, I.R. Vassiliev, D.R. Bryant, A.D. Jones, J.H. Golbeck, P.R. Chitnis, Recruitment of a foreign quinone into the A1 site of photosystem I. I. Genetic and physiological characterization of phyloquinone biosynthetic pathway mutants in *Synechocystis* sp. PCC 6803, *J. Biol. Chem.* 275 (2000) 8523–8530.
- [11] J. Soll, α -Tocopherol and plastoquinone synthesis in chloroplast membranes, *Methods Enzymol.* 148 (1987) 383–392.
- [12] M.J. Fryer, The antioxidant effects of thylakoid vitamin E (α -tocopherol), *Plant Cell Environ.* 15 (1992) 381–392.
- [13] C. Liljenberg, Characterization and properties of a protochlorophyllide ester in leaves of dark grown barley with geranylgeraniol as esterifying alcohol, *Physiol. Plant.* 32 (1974) 208–213.
- [14] A.R. Wellburn, Evidence for chlorophyll esterified with geranylgeraniol in newly greened leaves, *Biochem. Physiol. Pflanzen* 169 (1976) 265–271.
- [15] E. Walter, J. Schreiber, E. Zass, A. Eschenmoser, Bacteriochlorophyll a_{gg} and bacteriopheophytin a_p in photosynthetic reaction centers from *Rhodospirillum rubrum* G-9+, *Helv. Chim. Acta* 62 (1979) 899–920.
- [16] A. Henry, R. Powls, J.F. Pennock, *Scenedesmus obliquus* PS28: a tocopherol-free mutant which cannot form phytol, *Biochem. Soc. Trans.* 14 (1986) 958–959.
- [17] D.W. Bollivar, S. Wang, J.P. Allen, C.E. Bauer, Molecular genetic analysis of terminal steps in bacteriochlorophyll *a* biosynthesis: characterization of a *Rhodobacter capsulatus* strain that synthesizes geranylgeraniol-esterified bacteriochlorophyll *a*, *Biochemistry* 33 (1994) 12763–12768.
- [18] R. Tanaka, U. Oster, E. Kruse, W. Rüdiger, B. Grimm, Reduced activity of geranylgeranyl reductase leads to loss of chlorophyll and tocopherol and to partially geranylgeranylated chlorophyll in transgenic tobacco plants expressing antisense RNA for geranylgeranyl reductase, *Plant Physiol.* 120 (1999) 695–704.
- [19] T. Grasses, B. Grimm, O. Koroleva, P. Jahns, Loss of α -tocopherol in tobacco plants with decreased geranylgeranyl reductase activity does not modify photosynthesis in optimal growth conditions but increases sensitivity to high-light stress, *Planta* 213 (2001) 620–628.
- [20] M. Havaux, C. Lütz, B. Grimm, Chloroplast membrane photostability in *chlP* transgenic tobacco plants deficient in tocopherols, *Plant Physiol.* 132 (2003) 300–310.
- [21] H.A. Addlesee, L.C.D. Gibson, P.E. Jensen, C.N. Hunter, Cloning, sequencing and functional assignment of the chlorophyll biosynthesis gene, *chlP*, of *Synechocystis* sp. PCC 6803, *FEBS Lett.* 389 (1996) 126–130.

- [22] R. Rippka, J. Deruelles, J.B. Waterbury, M. Herdman, R.Y. Stanier, Generic assignments, strain histories and properties of pure cultures of cyanobacteria, *J. Gen. Microbiol.* 111 (1979) 1–61.
- [23] C. Yanisch-Perron, J. Vieira, J. Messing, Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors, *Gene* 33 (1985) 103–199.
- [24] H.K. Lichtenthaler, Chlorophylls and carotenoids: pigments of photosynthetic biomembranes, *Methods Enzymol.* 148 (1987) 350–382.
- [25] R.M. Mannan, H.B. Pakrasi, Dark heterotrophic growth-conditions result in an increase in the content of photosystem-II units in the filamentous cyanobacterium *Anabaena variabilis* ATCC-29413, *Plant Physiol.* 103 (1993) 971–977.
- [26] E. Collakova, D. DellaPenna, Isolation and functional analysis of homogenisate phytyltransferase from *Synechocystis* sp. PCC 6803 and *Arabidopsis*, *Plant Physiol.* 127 (2001) 1113–1124.
- [27] J. Yu, Q. Wu, H. Mao, N. Zhao, W.F. Vermaas, Effects of chlorophyll availability on phycobilisomes in *Synechocystis* sp. PCC 6803, *IUBMB Life* 48 (1999) 625–630.
- [28] G. Shen, S. Boussiba, W.F. Vermaas, *Synechocystis* sp. PCC 6803 strains lacking photosystem I and phycobilisome function, *Plant Cell* 5 (1993) 1853–1863.
- [29] S.A. Walter, Phycobilisome and phycobiliprotein structure, in: D.A. Bryant (Ed.), *The Molecular Biology of Cyanobacteria*, Kluwer Academic Publishers, Dordrecht, 1994, pp. 139–216.
- [30] E. Gantt, C.A. Lipschultz, J. Grabowski, B.K. Zimmermann, Phycobilisomes from blue-green and red algae. Isolation criteria and dissociation characteristics, *Plant Physiol.* 63 (1979) 615–620.
- [31] G. Renger, Studies about the mechanism of herbicidal interaction with photosystem II in isolated chloroplasts, *Z. Naturforsch.* 34c (1979) 1010–1014.
- [32] M. Schledz, A. Seidler, P. Beyer, G. Neuhaus, A novel phytyltransferase from *Synechocystis* sp. PCC 6803 involved in tocopherol biosynthesis, *FEBS Lett.* 499 (2001) 15–50.
- [33] M. Shibata, T. Mikota, A. Yoshimura, N. Iwata, M. Tsuyama, Y. Kobayashi, Chlorophyll formation and photosynthetic activity in rice mutants with alterations in hydrogenation of the chlorophyll alcohol side chain, *Plant Sci.* 166 (2004) 593–600.