



Mutation of Gly195 of the ChlH Subunit of Mg-chelatase Reduces Chlorophyll and Further Disrupts PS II Assembly in a Ycf48-Deficient Strain of *Synechocystis* sp. PCC 6803

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Biogenesis of the photosystems in oxygenic phototrophs requires co-translational insertion of chlorophyll *a*. The first committed step of chlorophyll *a* biosynthesis is the insertion of a Mg²⁺ ion into the tetrapyrrole intermediate protoporphyrin IX, catalyzed by Mg-chelatase. We have identified a *Synechocystis* sp. PCC 6803 strain with a spontaneous mutation in *chlH* that results in a Gly195 to Glu substitution in a conserved region of the catalytic subunit of Mg-chelatase. Mutant strains containing the ChlH Gly195 to Glu mutation were generated using a two-step protocol that introduced the *chlH* gene into a putative neutral site in the chromosome prior to deletion of the native gene. The Gly195 to Glu mutation resulted in strains with decreased chlorophyll *a*. Deletion of the PS II assembly factor Ycf48 in a strain carrying the ChlH Gly195 to Glu mutation did not grow photoautotrophically. In addition, the ChlH-G195E:ΔYcf48 strain showed impaired PS II activity and decreased assembly of PS II centers in comparison to a ΔYcf48 strain. We suggest decreased chlorophyll in the ChlH-G195E mutant provides a background to screen for the role of assembly factors that are not essential under optimal growth conditions.

Keywords: ChlH, Ycf48, chlorophyll biosynthesis, PS II, *Synechocystis*

INTRODUCTION

In oxygenic photosynthesis Photosystem II (PS II) and Photosystem I (PS I) catalyze the conversion of light energy into the chemical energy that is required for carbon fixation. The reaction center (RC) and core antenna proteins of both PS II and PS I require the co-translational insertion of chlorophyll *a* for correct folding (Eichacker et al., 1996; Sobotka, 2014; Yang et al., 2015). In addition, the co-ordinated production of chlorophyll and chlorophyll-binding proteins is essential to prevent the accumulation of potentially detrimental unbound chlorophyll and chlorophyll precursors. Although a large proportion of newly synthesized chlorophyll *a* is associated with PS I, the assembly of PS II and chlorophyll biosynthesis are synchronized (He and Vermaas, 1998). There are 70 chlorophyll *a* molecules in the PS II pigment-protein complex along with at least 20 protein subunits and additional cofactors (Umena et al., 2011; Shen, 2015). Assembly of PS II involves the formation of four pre-complexes, each containing one of the four chlorophyll *a*-binding proteins

that are found in the mature complex: D1 and D2 that form the RC, and CP43 and CP47 that function as a core antenna (Boehm et al., 2011). The pre-complexes are assembled, beginning with the association of the D1- and D2-containing modules, to form larger assembly intermediates and finally the mature photosystem (Komenda et al., 2012). In addition, the D1 subunit is the site of light-induced photo-oxidative damage and a repair cycle enables the removal and replacement of damaged D1 to maintain PS II activity (Komenda et al., 2012). Part of this repair cycle involves the removal of chlorophyll *a* from damaged D1, as well as insertion of chlorophyll *a* into new D1 subunits (Yao et al., 2012).

The chlorophyll *a* biosynthesis pathway is comprised of at least 15 enzymatic steps, many of which are shared with the biosynthetic pathway for heme (Sobotka, 2014; Fujita et al., 2015). The first dedicated step in the synthesis of chlorophyll *a* is the insertion of Mg²⁺ into protoporphyrin IX (PP IX) to form Mg-PP IX; this reaction is catalyzed by Mg-chelatase (Jensen et al., 1996). This represents a branch point that contributes to the regulation of tetrapyrrole biosynthesis by controlling the partitioning of precursor molecules such as PP IX into different biosynthetic pathways (Shepherd et al., 2005). Mg-chelatase is a large protein complex consisting of three different subunits CHLI, CHLD, and CHLH. Both CHLI and CHLD form hexamers that associate with a single catalytic CHLH subunit (Wang and Grimm, 2015). The corresponding ChlH protein in cyanobacteria is comprised of an N-terminal 'head' domain and a larger domain that forms a hollow, cage-like structure that binds porphyrin and a similar domain organization is expected in plant CHLH (Qian et al., 2012; Chen et al., 2015). Regulation of the Mg-chelatase enzyme occurs through association of the CHLH subunit with the GUN4 protein, in an interaction that stimulates enzyme activity (Larkin et al., 2003; Sobotka et al., 2008). In addition, in plants, CHLH is involved in plastid-nuclear signaling and in cyanobacteria ChlH has a regulatory role as an anti-sigma factor, binding to the RNA polymerase sigma factor SigE in the light (Mochizuki et al., 2001; Osanai et al., 2009). Two mutant lines of *Arabidopsis thaliana* with mutations in CHLH have been described: the Pro to Leu mutation in *cch* plants corresponds to the Pro595 residue in ChlH from the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) and the Ala to Val substitution in the *gun5* mutant corresponds to the Ala942 residue. Both Pro595 and Ala942 are located in the central cage-like structure of the protein and may interfere with the chelation reaction due to spatial hindrance (Chen et al., 2015). In *A. thaliana* these mutations caused reductions in chlorophyll levels of ~70 and ~30% in the *cch* and *gun5* mutants, respectively (Mochizuki et al., 2001).

Re-sequencing of *Synechocystis* 6803 has shown genetic differences have arisen between wild-type sub-strains under laboratory conditions (Jones, 2014). This occurrence of spontaneous mutations has produced several mutations in a sub-strain, GT-O2, including in the *chlH* gene (Morris et al., 2014). The mutation in *chlH* results in a Gly195 to Glu amino acid substitution in a conserved region of the ChlH protein. The GT-O2 strain was able to grow photoautotrophically,

although at a slightly slower rate and reaching a lower OD_{730 nm} compared to its parental strain, GT-O1 (Morris et al., 2014). In this report, we show the construction and characterization of *Synechocystis* 6803 strains carrying this ChlH Gly195 to Glu mutation. In addition, we investigated the impact of impairing biogenesis of PS II in the ChlH mutant by deleting the assembly factor Ycf48. We selected the Ycf48 protein [HCF136 in *A. thaliana* and the first assembly factor discovered (Meurer et al., 1998)] because it is transiently associated with the D1 pre-complex during assembly and repair of PS II. This factor binds to the D1 precursor polypeptide (pD1) and stabilizes formation of the RC II complex containing the D1 and D2 pre-complex modules (Komenda et al., 2008; Mabbitt et al., 2014). We hypothesized that deletion of Ycf48 in a strain where chlorophyll *a* supply is reduced may impact on both PS II biogenesis and the PS II repair cycle.

MATERIALS AND METHODS

Synechocystis 6803 Growth Conditions and Construction of Mutant Strains

Strains of *Synechocystis* 6803 were maintained on BG-11 medium agar plates supplemented with 5 mM glucose, 20 μM atrazine, 10 mM TES-NaOH (pH 8.2), 0.3% sodium thiosulfate and appropriate antibiotics. Liquid cultures were grown photoautotrophically or photomixotrophically (with 5 mM glucose) in BG-11 liquid medium with appropriate antibiotics, as previously described (Eaton-Rye, 2011; Summerfield et al., 2013).

Generation of *chlH* mutants was performed in two steps. Amplicons were generated using overlap-extension PCR (Bryskin and Matsumura, 2010), in which most of the *slr0168* gene was deleted and replaced by a spectinomycin-resistance cassette (Supplementary Figures S1A,B). The *slr0168* gene site has been used as a neutral site for integration of genes into the chromosome previously with no reported effect on phenotype (Kunert et al., 2000). A HindIII restriction site was introduced into the 5' end of the spectinomycin-resistance cassette using PCR. This amplicon was ligated into pGEM-T Easy and the resulting Δ*slr0168*:specR plasmid was transformed into wild-type *Synechocystis* 6803 (GT-O1) to create the Δ*slr0168* neutral site control strain (Supplementary Figure S1B). The *slr1055* (*chlH*) gene was amplified from the GT-O1 and GT-O2 wild types (Morris et al., 2014), using primers designed to introduce HindIII restriction sites at the 5' and 3' ends of the amplicons (Supplementary Table S1). These amplicons and the Δ*slr0168*:specR plasmid, were digested with HindIII and ligated to produce the ChlH-G195G:specR and ChlH-G195E:specR plasmids, respectively. The resultant plasmids were transformed into *Synechocystis* 6803 strains (GT-O1 or GT-O2) to introduce either the unmodified *chlH* (in the GT-O1:G195G and GT-O2:E195G strains) or the mutant *chlH* gene (in the GT-O1:G195E and GT-O2:E195E strains) into the putative neutral site (Supplementary Figure S1). In the second step, a Δ*chlH* plasmid was constructed using overlap-extension

PCR in which a chloramphenicol-resistance cassette was located between the sequences up- and downstream of the native *chlH* gene (Supplementary Figures S1C,D). This plasmid was transformed into the strains which contained a copy of the *chlH* gene in the neutral site to produce strains which each contained only one copy of *chlH*, either the GT-O1 (Gly195) or GT-O2 (Gly195 to Glu) variant. A summary of the mutants created in this study is presented in **Table 1**.

Construction of the $\Delta ycf48$ ($\Delta slr2034$) plasmid has been described previously (Jackson et al., 2014); this plasmid was transformed into the GT-O1:G195G and GT-O1:G195E strains to produce the GT-O1:G195G: $\Delta Ycf48$ and GT-O1:G195E: $\Delta Ycf48$ strains, respectively. Colony PCR and sequencing were used to confirm the mutant genotypes as appropriate (data not shown).

Physiological Measurements

Photoautotrophic growth, whole-cell absorption spectra, oxygen evolution, and chlorophyll *a* fluorescence analyses were performed as previously described (Crawford et al., 2016). Cells grown photomixotrophically to mid-exponential phase were washed and re-suspended to an optical density at 730 nm ($OD_{730\text{ nm}}$) of 1.5 in BG-11 supplemented with 25 mM HEPES-NaOH (pH 7.5) for physiological measurements. Chlorophyll *a* was quantified by extraction in methanol and measurement at 663 nm (MacKinney, 1941). Low-temperature (77 K) fluorescence emission spectroscopy was performed using sodium fluorescein as an internal standard as previously described (Crawford et al., 2016). Traces were normalized to the fluorescein emission maxima at 505 nm.

Protein Analyses

For analysis of PS II assembly, thylakoid membranes were isolated from photomixotrophically grown cultures, solubilized with β -dodecylmaltoside and separated by blue-native polyacrylamide gel electrophoresis (BN-PAGE) as previously described (Crawford et al., 2016). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes for 1 h at 25 V in the presence of 0.1% SDS, and were subjected to immunodetection using antibodies to PS II and PS I core protein subunits (Jackson and Eaton-Rye, 2015).

RESULTS

A Strain with an Altered Whole-Cell Spectrum Had Accumulated Several Mutations Including a *chlH* Mutation Changing Residue Gly195 to Glu

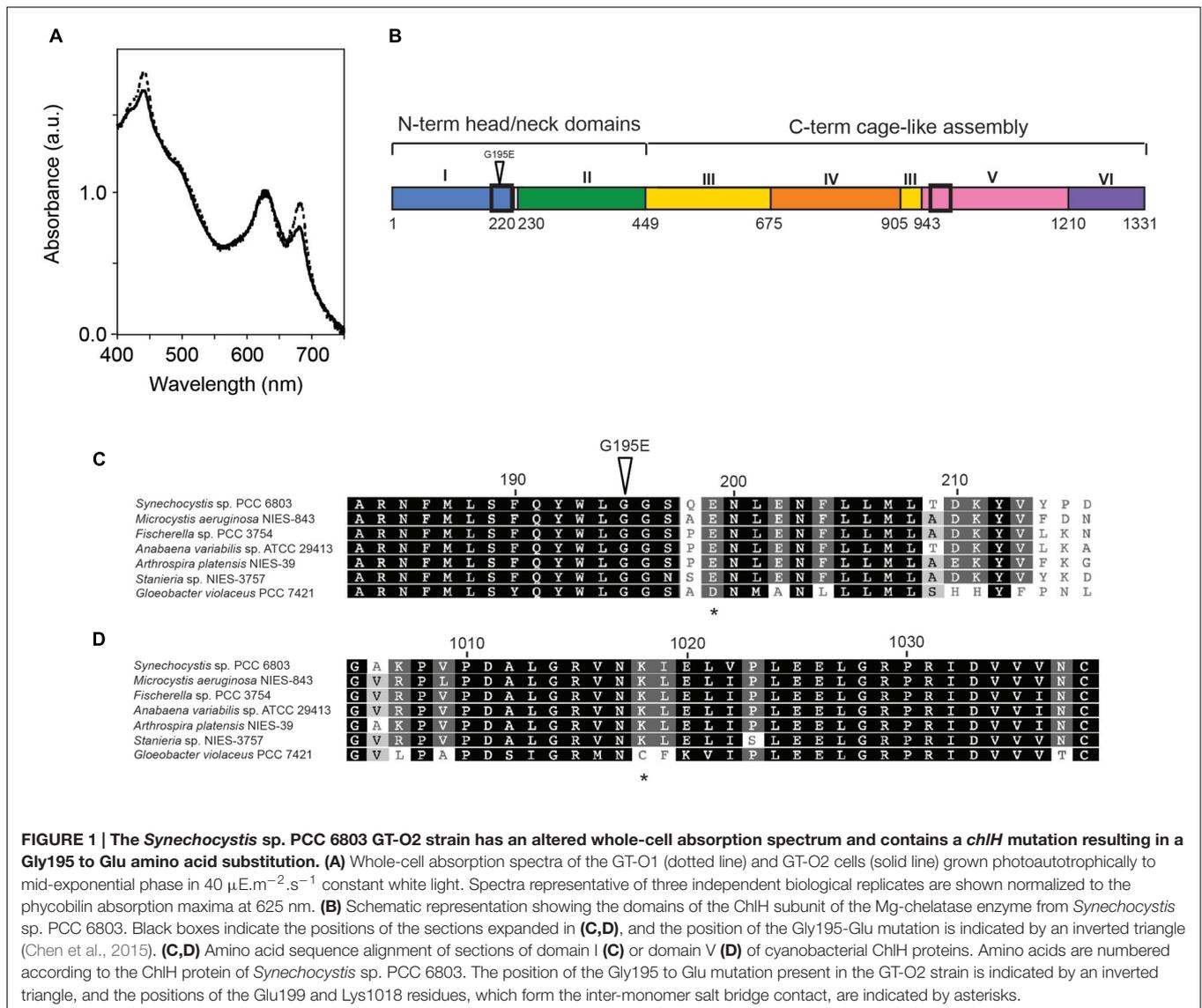
Comparison of two strains in our laboratories, GT-O1 and GT-O2, showed differences in whole-cell absorption spectra. Both strains exhibited absorption maxima at 625 nm, as well as at 435 and 685 nm, corresponding to phycobilins and chlorophyll *a*, respectively (**Figure 1A**). The GT-O2 strain exhibited a reduction in the peaks at 435 nm and 685 nm corresponding to chlorophyll *a* absorbance but not the 625 nm phycobilin peak (**Figure 1A**). Six unique genome sequence variants are present in the GT-O2 strain compared to GT-O1 cells (Morris et al., 2014). These are predicted to result in amino acid changes in five proteins: the long-chain fatty acid CoA ligase, FadD (Slr1609, Arg641 to Gln); the serine metalloprotease, HtrA/DegP (Slr1204, frameshift after amino acid 66 of 452); hypothetical protein Slr0154 (Ser307 to Phe); the histidine kinase, Hik8 (Slr0750, Arg65 to Cys), and the Mg-chelatase catalytic subunit, ChlH (Slr1055, Gly195 to Glu). The Mg-chelatase enzyme catalyzes the first committed step in chlorophyll *a* biosynthesis, therefore the ChlH Gly195 to Glu mutation was a candidate for the decreased absorbance of chlorophyll *a* in the GT-O2 wild type.

The ChlH Gly195 residue is located in the $\alpha 6$ helix of the N-terminal 'head' domain I (**Figure 1B**; Supplementary Figure S2). This Gly195 residue was conserved in ChlH proteins representing all five sections of cyanobacteria, and is part of a conserved protein region (**Figure 1C**). Although chiefly monomeric in solution, the crystal structure of ChlH obtained by Chen et al. (2015) indicated that this protein can form a dimer, and that interactions occur exclusively between the N-terminal domain I and the domain V of opposing monomers (Supplementary Figure S2). Dimerization of ChlH subunits is mediated by polar interactions, including six hydrogen bonds and a salt bridge between the Glu199 and Lys1018 residues of opposing monomers. The Gly195 residue is in close proximity to Glu199 (Supplementary Figure S2); the Glu199 residue is conserved in many cyanobacteria, but is substituted for an Asp in the cyanobacterium *Gloeobacter violaceus* PCC 7421. The Lys1018 residue was found in the aligned cyanobacterial ChlH protein sequences except that of *G. violaceus* PCC 7421 (**Figure 1D**).

TABLE 1 | Strains of *Synechocystis* sp. PCC 6803 used in this study.

Strain	Background	Insertion at neutralsite (<i>slr0168</i>)	Gene at native <i>chlH</i> site (<i>slr1055</i>)	Reference
GT-O1	–	–	<i>chlH</i> (G195)	Morris et al., 2014
GT-O2	–	–	<i>chlH</i> (E195)	Morris et al., 2014
$\Delta slr0168$	GT-O1	spec ^R	<i>chlH</i> (G195)	This study
GT-O1:G195G	GT-O1	<i>chlH</i> (G195) + spec ^R	cam ^R ($\Delta chlH$)	This study
GT-O1:G195E	GT-O1	<i>chlH</i> (E195) + spec ^R	cam ^R ($\Delta chlH$)	This study
GT-O2:E195G	GT-O2	<i>chlH</i> (G195) + spec ^R	cam ^R ($\Delta chlH$)	This study
GT-O2:E195E	GT-O2	<i>chlH</i> (E195) + spec ^R	cam ^R ($\Delta chlH$)	This study

spec^R and cam^R refer to spectinomycin-resistance and chloramphenicol-resistance cassettes, respectively.



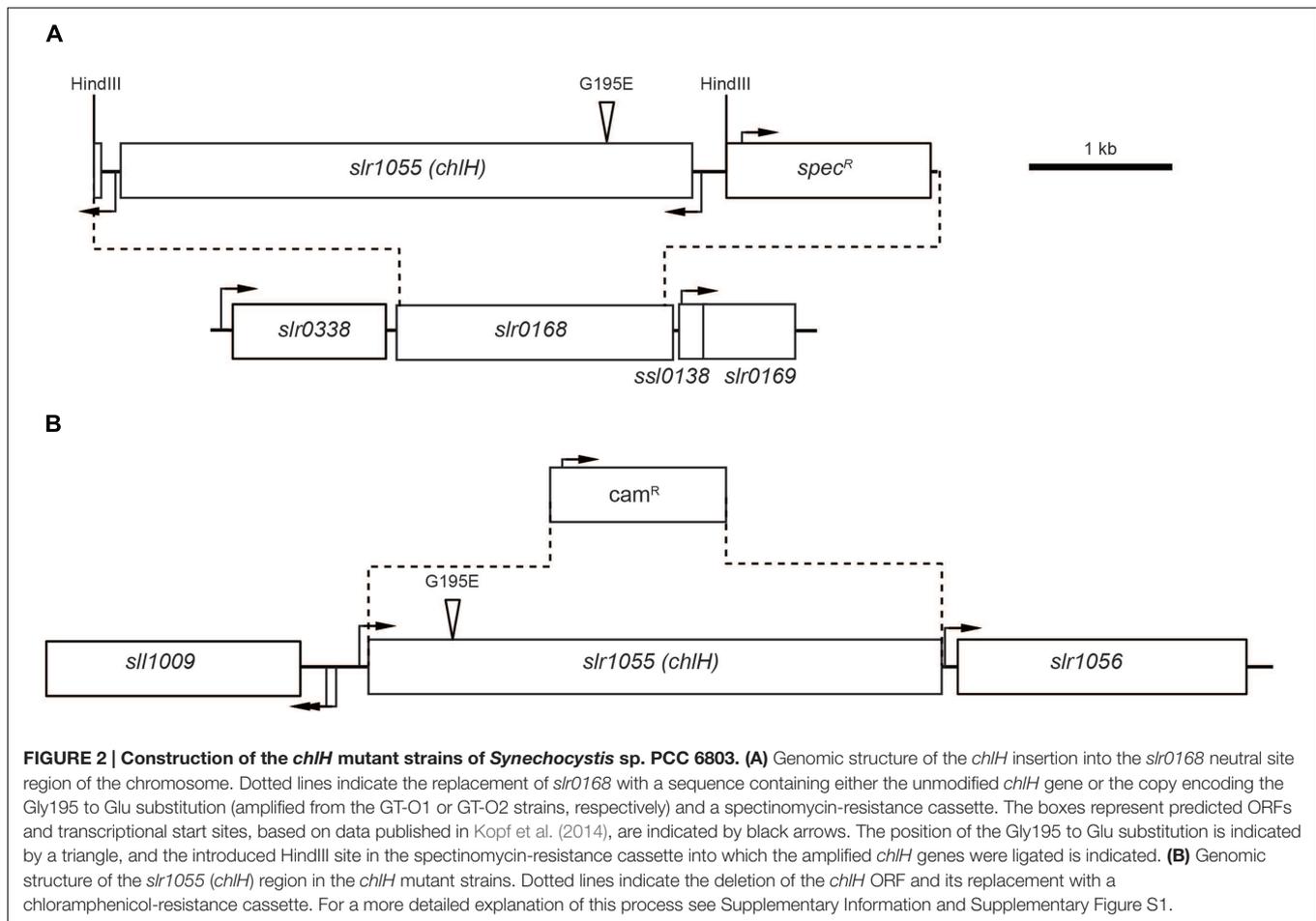
Generation of Mutations in *chlH* with a Two-Step Process Involving Insertion into the Putative Neutral Site at *slr0168*

To determine the effect of the ChlH Gly195 to Glu mutation *in vivo*, a copy of either the unmodified gene (encoding Gly195) or mutant *chlH* gene (encoding the Gly195 to Glu mutation) was introduced at a putative neutral site (*slr0168*) in the *Synechocystis* 6803 genome (Kunert et al., 2000). A spectinomycin-resistance cassette inserted downstream of the *chlH* gene enabled selection (Figure 2A). Once the introduced *chlH* gene had been fully segregated, the native gene was deleted and a chloramphenicol-resistance cassette was inserted in place of this copy of *chlH* (Figure 2B). This method was used to generate strains containing only the GT-O1 copy of *chlH*, encoding Gly195, in both the GT-O1 and GT-O2 backgrounds. Similarly, strains were generated containing only the GT-O2 copy of *chlH*, encoding the Gly195 to Glu substitution, in both the GT-O1 or GT-O2 backgrounds.

Mutants in the GT-O2 background enabled us to determine the impact of restoring the Gly195 to this background, as well as, including an additional control strain where the Glu195 was introduced into ChlH in the neutral site. The sequences of the oligonucleotides used for the construction and verification of these strains are in Table S1. In addition, a $\Delta\textit{slr0168}$ control strain was produced which contained only the spectinomycin-resistance cassette in the neutral site and which retained the native *chlH* gene at its wild-type locus.

A Mutation That Changes ChlH Residue 195 from Gly to Glu Altered the Whole-Cell Spectrum

The whole-cell spectra of the GT-O1 wild type and $\Delta\textit{slr0168}$ control strain were similar (data not shown). Introduction of unmodified *chlH*, under its own promoter, to produce the GT-O1:G195G strain using the *slr0168* site resulted in a whole-cell

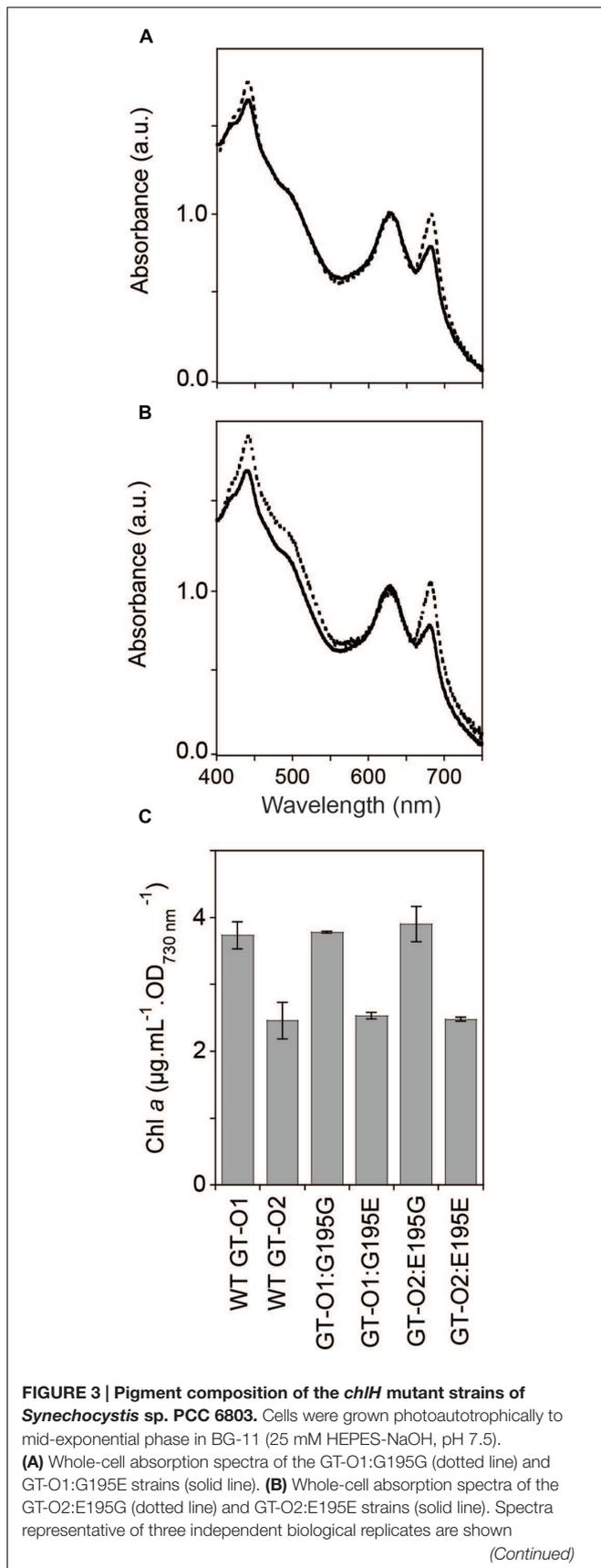


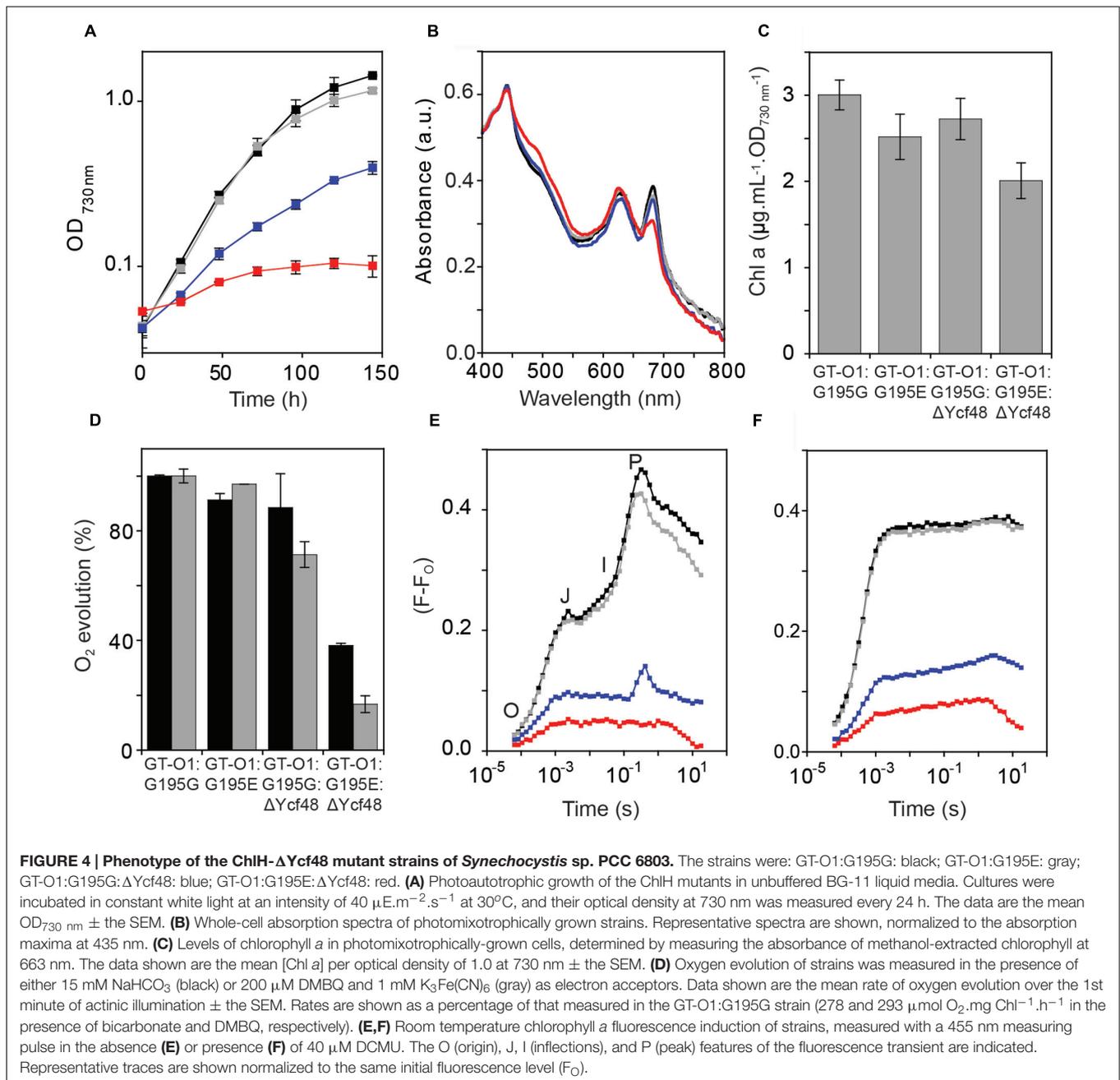
spectrum similar to that observed with GT-O1 cells, whereas introduction of the ChlH Gly195 to Glu mutation to produce the GT-O1:G195E strain resulted in decreased chlorophyll *a* absorption at both the 435 and 685 nm maxima compared to the GT-O1 strain (Figure 3A). Conversely, the introduction of unmodified *chlH* into the GT-O2 background resulted in the GT-O2:E195G strain which had increased chlorophyll *a* maxima that were similar to the GT-O1 wild type (cf. Figures 1A and 3B). In addition, the GT-O2:E195E strain showed an increased absorption in the 475–520 nm range compared to the GT-O2:E195G strain suggesting elevated carotenoid levels. Furthermore, the spectrum of the GT-O2:E195E strain was similar to spectra from the GT-O2 and GT-O1:G195E strains, suggesting that the presence of the Gly195 to Glu variant in ChlH results in a decrease in chlorophyll *a* absorbance (Figures 1A and 3A,B). The quantification of methanol-extracted chlorophyll *a* showed that the Gly195 to Glu mutation in ChlH resulted in a ~35–40% reduction of chlorophyll *a* accumulation in the GT-O2 and the GT-O1:G195E and GT-O2:E195E strains, in comparison to the GT-O1 and the GT-O1:G195G and GT-O2:E195G strains in samples normalized to an OD_{730 nm} of 1.0 (Figure 3C). The Gly195 to Glu mutation had little effect on the rate of photoautotrophic growth of the GT-O1:G195E strain, although these cells reached a slightly lower final OD_{730 nm} in stationary

phase (Figure 4A); this is consistent with that observed in the GT-O2 wild type (Morris et al., 2014). The photoautotrophic growth of the $\Delta slr0168$ control strain was indistinguishable from the GT-O1 wild type and the GT-O1:G195G strain (data not shown). Using flow cytometry and electron microscopy we did not detect any difference in size between strains containing Gly195 or Glu195 (data not shown), therefore the OD_{730 nm} should represent a similar cell number in all strains compared.

Introduction of the ChlH Gly195 to Glu Mutation Abolished Photoautotrophic Growth in a $\Delta Ycf48$ Strain

Formation of mature PS II is a multistep process involving the co-ordinated assembly of several intermediate complexes that require the insertion of chlorophyll *a*. The Ycf48 assembly factor is associated with the RC pre-complex; however, the absence of this factor does not completely block PS II assembly (Komenda et al., 2008; Jackson et al., 2014; Sobotka, 2014). We determined whether deletion of Ycf48 in a strain with reduced chlorophyll *a* would impact on PS II biogenesis and the PS II repair cycle. Deletion of Ycf48 resulted in reduced photoautotrophic growth, with a decrease in the final OD_{730 nm} reached in stationary phase from 1.43 ± 0.02 in the GT-O1:G195G background to



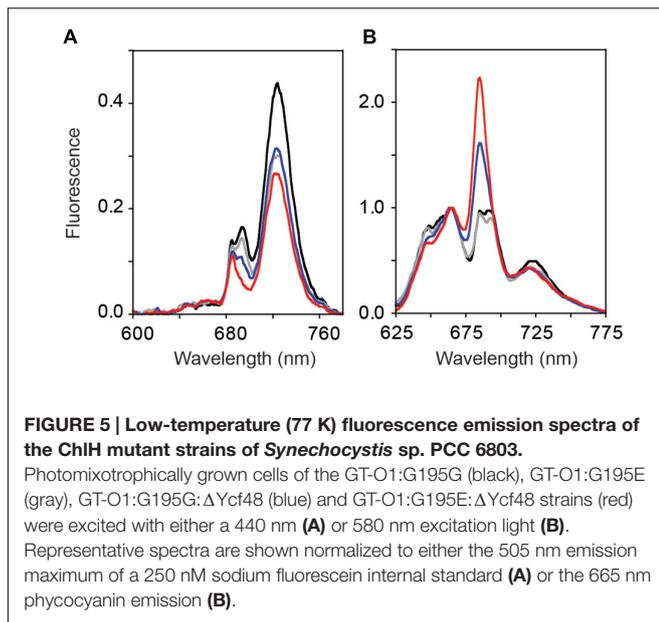


removal of Ycf48 resulted in a considerable decrease in the variable fluorescence level and this was observed in the GT-O1:G195G: Δ Ycf48 strain (Jackson et al., 2014). The variable fluorescence induction of the GT-O1:G195E: Δ Ycf48 strain was further reduced compared to the GT-O1:G195G: Δ Ycf48 strain and did not display an I-P rise (Figure 4E). In the presence of 40 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which blocks electron transfer beyond Q_A of PS II, variable fluorescence was similar in the GT-O1:G195G and GT-O1:G195E strains, reduced in the GT-O1:G195G:Ycf48 strain and further decreased in the GT-O1:G195E: Δ Ycf48 strain (Figure 4F). Variable fluorescence in the presence of DCMU provides an

indication of the numbers of assembled PS II centers and these data are consistent with decreased centers in both strains lacking Ycf48, with the lowest number of centers in the GT-O1:G195E: Δ Ycf48 strain.

The 77 K Fluorescence Emission Spectra Were Altered in the GT-O1:G195E: Δ Ycf48 Mutant

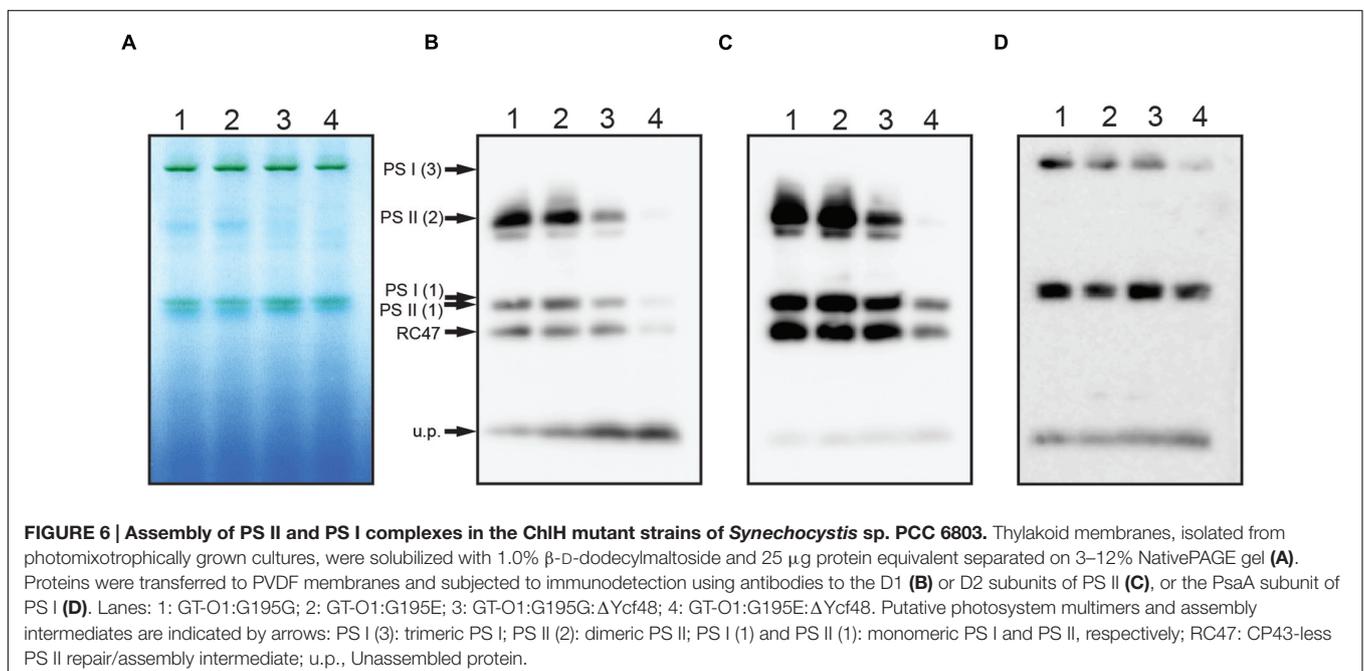
Reduced PS II-specific oxygen evolution and variable fluorescence in the GT-O1:G195G: Δ Ycf48 and the GT-O1:G195E: Δ Ycf48 strains are consistent with decreased numbers



of assembled PS II centers in these strains. Low-temperature (77 K) fluorescence emission spectroscopy was performed on photomixotrophically grown cultures using 250 nM sodium fluorescein as an internal standard. Introduction of the ChlH Gly195 to Glu mutation had little effect on the 685 and 695 nm fluorescence emission in the GT-O1:G195E strain with a 440 nm excitation light (Figure 5A). The 685 nm emission peak is attributed to unassembled pre-complexes and the CP43 core antenna protein, while the emission at 695 nm arises from the CP47 core antenna and is indicative of fully assembled PS II RCs (Boehm et al., 2011; D'Haene et al., 2015). The fluorescence

emission maximum observed at 725 nm originates from PS I, and this was decreased by ~35% in the GT-O1:G195E strain compared with the GT-O1:G195G strain when spectra were normalized to the emission maximum of fluorescein at 505 nm (Figure 5A). A similar decrease in the emission at 725 nm was observed in the GT-O1:G195G:ΔYcf48 strain and this strain also displayed a decreased emission at 685 nm with a further decrease at 695 nm. This is consistent with impaired assembly and/or repair of PS II in the absence of Ycf48; however, the decrease in fluorescence emission from PS I suggests an additional effect on PS I assembly due to the removal of this assembly factor. Deletion of Ycf48 in a ChlH-G195E background resulted in a complete absence of the 695 nm emission in the GT-O1:G195E:ΔYcf48 strain, suggesting a decreased number of PS II centers; this strain also showed a decreased PS I emission maxima at 725 nm, compared to the GT-O1:G195G:ΔYcf48 strain.

Steady-state phycobilisome-coupled energy transfer was assessed by low-temperature emission spectra following excitation at 580 nm. When normalized to the fluorescence emission maximum at 665 nm, little difference was observed between the emission spectra of the GT-O1:G195G and GT-O1:G195E strains, except for a slight decrease in the 725 nm emission originating from PS I in the latter, consistent with the decrease in PS I emissions observed in 440 nm-excited cells (Figure 5B). In the GT-O1:G195G:ΔYcf48 strain, an increase in the fluorescence emission at 685 nm was observed; this emission originates from the ApcE terminal emitter of the phycobilisome antenna and indicates an increased level of uncoupled phycobilisomes in this strain (Shen et al., 1993; Jackson et al., 2014). This emission was further increased in the GT-O1:G195E:ΔYcf48 strain, and both this and the GT-O1:G195G:ΔYcf48 strains displayed the decreased PS I emission seen in the GT-O1:G195E strain.



BN-PAGE Confirmed PS II and PS I Assembly was Altered in the GT-O1:G195E:ΔYcf48 Mutant

To further analyze the PS II complexes in the ChlH mutant strains, thylakoid membranes were analyzed using BN-PAGE and western blotting. In the GT-O1:G195G and GT-O1:G195E strains, the D1 and D2 core subunits were detected at similar levels in dimeric and monomeric PS II complexes, and in a CP43-less assembly/repair intermediate, RC47 (Figures 6B,C). In the GT-O1:G195G:ΔYcf48 strain the D1 and D2 proteins detected in PS II dimers and monomers were reduced, and the unassembled D1 slightly increased. However, only low levels of PS II monomers and only trace amounts of dimers were detected in the GT-O1:G195E:ΔYcf48 strain, consistent with the very low levels of PS II activity, while a low level of the RC47 complex was present. In addition, an antibody to the PsaA subunit of PS I revealed that the ratio of PS I monomers to trimers in the GT-O1:G195G:ΔYcf48 appeared to increase and that trimers were greatly reduced or destabilized in the GT-O1:G195E:ΔYcf48 mutant (Figure 6D).

DISCUSSION

A Strain with the ChlH Gly195 to Glu Mutation has Less Chlorophyll *a*

The ChlH subunit of Mg-chelatase catalyzes the first committed step in chlorophyll *a* biosynthesis and is involved in the regulation of gene expression in cyanobacteria (Jensen et al., 1996; Osanai et al., 2009). Commensurate with the importance of ChlH, segregation of gene-targeted knockouts has not been successful (Kong and Xu, 2002). Instead we have used a two-step approach to construct mutant strains with an inactivated *chlH* locus but which contain a *chlH* gene, expressed from a neutral site and encoding either an unmodified ChlH protein or the Gly195 to Glu variant. Characterization of these mutants revealed that introduction of the Gly195 to Glu mutation decreased the accumulation of chlorophyll *a*, with a more pronounced decrease in photoautotrophically grown cells compared to mixotrophically grown cultures. Our approach is readily applicable to the *in vivo* study of other targeted mutants in *chlH* from *Synechocystis* 6803.

The ChlH-Pro595 to Leu variant (equivalent to the substitution in the *A. thaliana cch* mutant) and the ChlH-Ala942 to Val change (equivalent to the substitution in the *A. thaliana gun5* mutant) have both been studied in the *Synechocystis* ChlH subunit *in vitro* (Davison and Hunter, 2011). In the *cch* and *gun5* mutants the observed reduction in chlorophyll was attributed to defective Mg-PP IX synthesis (Mochizuki et al., 2001). Likewise, *in vitro* characterization of the recombinant *Synechocystis* 6803 ChlH proteins showed both mutations abolished Mg-chelatase activity; however, Mg-chelatase activity was partially restored with the addition of the Gun4 protein (Davison and Hunter,

2011). The crystal structure of *Synechocystis* ChlH reveals that Pro595 and Ala942 are in the central cage-like structure of the protein that houses the catalytic site (Chen et al., 2015). However, while the Gly195 to Glu substitution in our *Synechocystis* 6803 ChlH-G195E strains also resulted in reduced chlorophyll *a*, the Gly195 residue is not in the catalytic cage-like domain. Therefore it is not clear whether the reduction in chlorophyll *a* in our mutants is caused by impaired catalytic activity or by an indirect mechanism. The proximity of Gly195 to the inter-dimeric salt bridge formed by Glu199 and Lys1018 might mean the Gly195 to Glu substitution could affect the formation and stability of the ChlH dimer; however, the physiological relevance of the ChlH dimer in chlorophyll *a* biosynthesis is unknown. In addition, the residues involved in salt bridge formation were not conserved in all cyanobacteria.

Combining the ChlH-Gly195 to Glu Mutation in a ΔYcf48 Strain Abolishes Photoautotrophic Growth

During PS II biogenesis and repair Ycf48 is transiently associated with pD1-containing pre-complexes prior to D1 processing and subsequent formation of the RC47 assembly intermediate (Komenda et al., 2007, 2008, 2012). In this study when the absence of Ycf48 was combined with the ChlH Gly195 to Glu mutation, the resultant double mutant showed limited photoautotrophic growth, a decreased level of PS II activity and a decrease in levels of assembled PS II dimers, monomers and the RC47 assembly intermediate compared with the GT-O1:G195G:ΔYcf48 strain (Figures 4–6). We also observed that PS I assembly was destabilized in our GT-O1:G195E:ΔYcf48 strain, perhaps as an indirect consequence of perturbing nascent PS II assembly and repair complexes. A similar effect on PS I assembly in an *A. thaliana* mutant lacking HCF136 – the homolog of Ycf48 – has also been reported (Plücker et al., 2002).

Given the close relationship between chlorophyll biosynthesis and the assembly and repair of PS II (Vavilin et al., 2007; Yao et al., 2012; Chidgey et al., 2014; Knoppová et al., 2014) and PS I (Kopečná et al., 2013; Hollingshead et al., 2016) manipulating the supply of chlorophyll may reveal novel roles for assembly factors of both PS II and PS I as seen in our GT-O1:G195E:ΔYcf48 strain.

AUTHOR CONTRIBUTIONS

TC performed the experiments and wrote the first draft of the manuscript. TS and JE-R designed the experiments, wrote, and edited the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.01060>

REFERENCES

- Boehm, M., Romero, E., Reisinger, V., Yu, J., Komenda, J., Eichacker, L. A., et al. (2011). Investigating the early stages of Photosystem II assembly in *Synechocystis* sp. PCC 6803: isolation of CP47 and CP43 complexes. *J. Biol. Chem.* 286, 14812–14819. doi: 10.1074/jbc.M110.207944
- Bryskin, A. V., and Matsumura, I. (2010). Overlap extension PCR cloning: a simple and reliable way to create recombinant plasmids. *Biotechniques* 48, 463–465. doi: 10.2144/000113418
- Chen, X., Pu, H., Fang, Y., Wang, X., Zhao, S., Lin, Y., et al. (2015). Crystal structure of the catalytic subunit of magnesium chelatase. *Nat. Plants* 1, 15125–15129. doi: 10.1038/nplants.2015.125
- Chidgey, J. W., Linhartová, M., Komenda, J., Jackson, P. J., Dickman, M. J., Canniffe, D. P., et al. (2014). A cyanobacterial chlorophyll synthase-HliD complex associates with the Ycf39 protein and the YidC/Alb3 insertase. *Plant Cell* 26, 1–14. doi: 10.1105/tpc.114.124495
- Crawford, T. S., Hanning, K. R., Chua, J. P. S., Eaton-Rye, J. J., and Summerfield, T. S. (2016). Comparison of D1' and D1-containing PS II reaction centre complexes under different environmental conditions in *Synechocystis* sp. PCC 6803. *Plant Cell Environ.* 39, 1715–1726. doi: 10.1111/pce.12738
- D'Haene, S. E., Sobotka, R., Bučinská, L., Dekker, J. P., and Komenda, J. (2015). Interaction of the PsbH subunit with a chlorophyll bound to histidine 114 of CP47 is responsible for the red 77K fluorescence of Photosystem II. *Biochim. Biophys. Acta* 1847, 1327–1334. doi: 10.1016/j.bbabi.2015.07.003
- Davison, P. A., and Hunter, C. N. (2011). Abolition of magnesium chelatase activity by the *gun5* mutation and reversal by Gun4. *FEBS Lett.* 585, 183–186. doi: 10.1016/j.febslet.2010.11.037
- Eaton-Rye, J. J. (2011). Construction of gene interruptions and gene deletions in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *Methods Mol. Biol.* 684, 295–312. doi: 10.1007/978-1-60761-925-3_22
- Eichacker, L. A., Helfrich, M., Rüdiger, W., and Muller, B. (1996). Stabilization of chlorophyll *a*-binding apoproteins P700, CP47, CP43, D2, and D1 by chlorophyll *a* or Zn-phenophytin *a*. *J. Biol. Chem.* 271, 32174–32179. doi: 10.1074/jbc.271.50.32174
- Fujita, Y., Tsujimoto, R., and Aoki, R. (2015). Evolutionary aspects and regulation of tetrapyrrole biosynthesis in cyanobacteria under aerobic and anaerobic environments. *Life* 5, 1172–1203. doi: 10.3390/life5021172
- He, Q., and Vermaas, W. (1998). 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, 5830–5835. doi: 10.1073/pnas.95.10.5830
- Hollingshead, S., Kopečná, J., Armstrong, D. R., Bučinská, L., Jackson, P. J., Chen, G. E., et al. (2016). Synthesis of chlorophyll-binding proteins in a fully segregated *Dytc54* strain of the cyanobacterium *Synechocystis* sp. PCC 6803. *Front. Plant Sci.* 7:292. doi: 10.3389/fpls.2016.00292
- Jackson, S. A., and Eaton-Rye, J. J. (2015). Characterization of a *Synechocystis* sp. PCC 6803 double mutant lacking the CyanoP and Ycf48 proteins of Photosystem II. *Photosynth. Res.* 124, 217–229. doi: 10.1007/s11120-015-0122-0
- Jackson, S. A., Hervey, J. R. D., Dale, A. J., and Eaton-Rye, J. J. (2014). Removal of both Ycf48 and Psb27 in *Synechocystis* sp. PCC 6803 disrupts Photosystem II assembly and alters Q_A^- oxidation in the mature complex. *FEBS Lett.* 588, 3751–3760. doi: 10.1016/j.febslet.2014.08.024
- Jensen, P. E., Gibson, L. C., Henningsen, K. W., and Hunter, C. N. (1996). Expression of the *chlI*, *chlD*, and *chlH* genes from the cyanobacterium *Synechocystis* PCC6803 in *Escherichia coli* and demonstration that the three cognate proteins are required for magnesium-protoporphyrin chelatase activity. *J. Biol. Chem.* 271, 16662–16667. doi: 10.1074/jbc.271.28.16662
- Jones, P. R. (2014). Genetic instability in cyanobacteria - an elephant in the room? *Front. Bioeng. Biotechnol.* 2:12. doi: 10.3389/fbioe.2014.00012
- Knopková, J., Sobotka, R., Tichy, M., Yu, J., Konik, P., Halada, P., et al. (2014). Discovery of a chlorophyll binding protein complex involved in the early steps of Photosystem II assembly in *Synechocystis*. *Plant Cell* 26, 1200–1212. doi: 10.1105/tpc.114.123919
- Komenda, J., Kuviková, S., Granvogel, B., Eichacker, L. A., Diner, B. A., and Nixon, P. J. (2007). Cleavage after residue Ala352 in the C-terminal extension is an early step in the maturation of the D1 subunit of Photosystem II in *Synechocystis* PCC 6803. *Biochim. Biophys. Acta* 1767, 829–837. doi: 10.1016/j.bbabi.2007.01.005
- Komenda, J., Nickelsen, J., Tichy, M., Prášil, O., Eichacker, L. A., and Nixon, P. J. (2008). The cyanobacterial homologue of HCF136/YCF48 is a component of an early Photosystem II assembly complex and is important for both the efficient assembly and repair of Photosystem II in *Synechocystis* sp. PCC 6803. *J. Biol. Chem.* 283, 22390–22399. doi: 10.1074/jbc.M801917200
- Komenda, J., Sobotka, R., and Nixon, P. J. (2012). Assembling and maintaining the Photosystem II complex in chloroplasts and cyanobacteria. *Curr. Opin. Plant Biol.* 15, 245–251. doi: 10.1016/j.pbi.2012.01.017
- Kong, R., and Xu, X. (2002). Three-piece-ligation PCR and application in disruption of chlorophyll synthesis genes in *Synechocystis* sp. PCC 6803. *Curr. Microbiol.* 44, 241–245. doi: 10.1007/s00284-001-0032-6
- Kopečná, J., Sobotka, R., and Komenda, J. (2013). Inhibition of chlorophyll biosynthesis at the protochlorophyllide reduction step results in the parallel depletion of Photosystem I and Photosystem II in the cyanobacterium *Synechocystis* PCC 6803. *Planta* 237, 497–508. doi: 10.1007/s00425-012-1761-4
- Kopf, M., Kähn, S., Scholz, L., Matthiesen, J. K. F., Hess, W. R., and Voß, B. (2014). Comparative analysis of the primary transcriptome of *Synechocystis* sp. PCC 6803. *DNA Res.* 21, 537–539. doi: 10.1093/dnares/dsu018
- Kunert, A., Hagemann, M., and Erdmann, N. (2000). Construction of promoter probe vectors for *Synechocystis* sp. PCC 6803 using the light-emitting reporter systems Gfp and LuxAB. *J. Microbiol. Methods* 41, 185–194. doi: 10.1016/S0167-7012(00)00162-7
- Larkin, R. M., Alonso, J. M., Ecker, J. R., and Chory, J. (2003). GUN4, a regulator of chlorophyll synthesis and intracellular signaling. *Science* 299, 902–906. doi: 10.1126/science.1079978
- Mabbitt, P. D., Wilbanks, S. M., and Eaton-Rye, J. J. (2014). Structure and function of the hydrophilic Photosystem II assembly proteins: Psb27, Psb28 and Ycf48. *Plant Physiol. Biochem.* 81, 96–107. doi: 10.1016/j.plaphy.2014.02.013
- MacKinney, G. (1941). Absorption of light by chlorophyll solutions. *J. Biol. Chem.* 140, 315–322.
- Meurer, J., Plücker, H., Kowallik, K. V., and Westhoff, P. (1998). A nuclear-encoded protein of prokaryotic origin is essential for the stability of Photosystem II in *Arabidopsis thaliana*. *EMBO J.* 17, 5286–5297. doi: 10.1093/emboj/17.18.5286
- Mochizuki, N., Bruslan, J. A., Larkin, R., Nagatani, A., and Chory, J. (2001). *Arabidopsis* genomes uncoupled 5 (GUN5) mutant reveals the involvement of Mg-chelatase H subunit in plastid-to-nucleus signal transduction. *Proc. Natl. Acad. Sci. U.S.A.* 98, 2053–2058. doi: 10.1073/pnas.98.4.2053
- Morris, J. N., Crawford, T. S., Jeffs, A., Stockwell, P. A., Eaton-Rye, J. J., and Summerfield, T. C. (2014). Whole genome re-sequencing of two “wild-type” strains of the model cyanobacterium *Synechocystis* sp. PCC 6803. *N. Z. J. Bot.* 52, 36–47. doi: 10.1080/0028825X.2013.846267
- Osanai, T., Imashimizu, M., Seki, A., Sato, S., Tabata, S., and Imamura, S. (2009). ChlH, the H subunit of the Mg-chelatase, is an anti-sigma factor for SigE in *Synechocystis* sp. PCC 6803. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6860–6865. doi: 10.1073/pnas.0810040106
- Plücker, H., Müller, B., Grohmann, D., Westhoff, P., and Eichacker, L. A. (2002). The HCF136 protein is essential for assembly of the Photosystem II reaction center in *Arabidopsis thaliana*. *FEBS Lett.* 532, 85–90. doi: 10.1016/S0014-5793(02)03634-7
- Qian, P., Marklew, C. J., Davison, P. A., Brindley, A. A., Söderberg, C., Alkaradaghi, S., et al. (2012). Structure of the cyanobacterial magnesium chelatase H subunit determined by single particle reconstruction and small-angle X-ray scattering. *J. Biol. Chem.* 287, 4946–4956. doi: 10.1074/jbc.M111.308239
- Shen, G., Boussiba, S., and Vermaas, W. F. J. (1993). *Synechocystis* sp. PCC 6803 strains lacking Photosystem I and phycobilisome function. *Plant Cell* 5, 1853–1863. doi: 10.1105/tpc.5.12.1853
- Shen, J.-R. (2015). The structure of Photosystem II and the mechanism of water oxidation in photosynthesis. *Annu. Rev. Plant Biol.* 66, 23–48. doi: 10.1146/annurev-arplant-050312-120129
- Shepherd, M., McLean, S., and Hunter, C. N. (2005). Kinetic basis for linking the first two enzymes of chlorophyll biosynthesis. *FEBS J.* 272, 4532–4539. doi: 10.1111/j.1742-4658.2005.04873.x
- Sobotka, R. (2014). Making proteins green: biosynthesis of chlorophyll-binding proteins in cyanobacteria. *Photosynth. Res.* 119, 223–232. doi: 10.1007/s11120-013-9797-2
- Sobotka, R., Dühring, U., Komenda, J., Peter, E., Gardian, Z., Tichy, M., et al. (2008). Importance of the cyanobacterial Gun4 protein for chlorophyll metabolism and assembly of photosynthetic complexes. *J. Biol. Chem.* 283, 25794–25802. doi: 10.1074/jbc.M803787200

- Summerfield, T. C., Crawford, T. S., Young, R. D., Chua, J. P. S., MacDonald, R. L., Sherman, L. A., et al. (2013). Environmental pH affects photoautotrophic growth of *Synechocystis* sp. PCC 6803 strains carrying mutations in the luminal proteins of PS II. *Plant Cell Physiol.* 54, 859–874. doi: 10.1093/pcp/pct036
- Umena, Y., Kawakami, K., Shen, J.-R., and Kamiya, N. (2011). Crystal structure of oxygen-evolving Photosystem II at a resolution of 1.9 Å. *Nature* 473, 55–60. doi: 10.1038/nature09913
- Vavilin, D., Yao, D., and Vermaas, W. (2007). Small Cab-like proteins retard degradation of Photosystem II-associated chlorophyll in *Synechocystis* sp. PCC 6803. *J. Biol. Chem.* 282, 37660–37668. doi: 10.1074/jbc.M707133200
- Wang, P., and Grimm, B. (2015). Organization of chlorophyll biosynthesis and insertion of chlorophyll into the chlorophyll-binding proteins in chloroplasts. *Photosynth. Res.* 126, 189–202. doi: 10.1007/s11120-015-0154-5
- Yang, H., Liu, J., Wen, X., and Lu, C. (2015). Molecular mechanism of Photosystem I assembly in oxygenic organisms. *Biochim. Biophys. Acta* 1847, 838–848. doi: 10.1016/j.bbabi.2014.12.011
- Yao, D. C. I., Brune, D. C., Vavilin, D., and Vermaas, W. F. J. (2012). Photosystem II component lifetimes in the cyanobacterium *Synechocystis* sp. strain PCC 6803: small Cab-like proteins stabilize biosynthesis intermediates and affect early steps in chlorophyll synthesis. *J. Biol. Chem.* 287, 682–692. doi: 10.1074/jbc.M111.320994

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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