The Plant Cell, Vol. 26: 1200–1212, March 2014, www.plantcell.org

Discovery of a Chlorophyll Binding Protein Complex Involved in the Early Steps of Photosystem II Assembly in *Synechocystis*[W]

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Efficient assembly and repair of the oxygen-evolving photosystem II (PSII) complex is vital for maintaining photosynthetic activity in plants, algae, and cyanobacteria. How chlorophyll is delivered to PSII during assembly and how vulnerable assembly complexes are protected from photodamage are unknown. Here, we identify a chlorophyll and β-carotene binding protein complex in the cyanobacterium *Synechocystis* PCC 6803 important for formation of the D1/D2 reaction center assembly complex. It is composed of putative short-chain dehydrogenase/reductase Ycf39, encoded by the *slr0399* gene, and two members of the high-light-inducible protein (Hlip) family, HliC and HliD, which are small membrane proteins related to the light-harvesting chlorophyll binding complexes found in plants. Perturbed chlorophyll recycling in a Ycf39-null mutant and copurification of chlorophyll synthase and unassembled D1 with the Ycf39-Hlip complex indicate a role in the delivery of chlorophyll to newly synthesized D1. Sequence similarities suggest the presence of a related complex in chloroplasts.

INTRODUCTION

Photosystem II (PSII) embedded in the thylakoid membranes of plants, algae, and cyanobacteria is the multisubunit enzyme complex that uses light energy to catalyze the highly energetically demanding reaction of water splitting. Recent three-dimensional structures of active dimeric PSII complexes from cyanobacteria have revealed the spatial organization of the protein subunits and cofactors involved in excitation energy transfer and electron transport (Ferreira et al., 2004; Guskov et al., 2009; Umena et al., 2011). Within the core of the complex there are two reaction center (RC) subunits called D1 and D2, which bind the cofactors involved in primary charge separation (Rappaport and Diner, 2008). On either side are CP43 and CP47, which both bind chlorophyll *a* and β-carotene and serve as inner light-harvesting complexes or antennae. On the periphery of these large subunits are 13 small, mostly single helix transmembrane subunits, while the lumenal part of the complex is shielded by the PsbO, PsbU, and PsbV extrinsic subunits (reviewed in Roose et al., 2007).

PSII biogenesis in the cyanobacterium *Synechocystis* sp PCC 6803 (hereafter, *Synechocystis*) occurs in a stepwise fashion from four preassembled smaller subcomplexes or modules (Komenda et al., 2012a). Each module consists of one large chlorophyll binding subunit (D1, D2, CP47, and CP43) and several neighboring low molecular mass membrane polypeptides plus bound pigment and other cofactors. PSII assembly is initiated through interaction of the D1 and D2 modules to form a RC intermediate, termed the RCII complex. The CP47 module (Boehm et al., 2011) is then attached to RCII forming a CP43-less complex, called RC47 (Boehm et al., 2012a). Oxygen-evolving monomeric and dimeric PSII are formed after addition of the CP43 module (Boehm et al., 2011), attachment of the luminal extrinsic proteins, and light-driven assembly of the oxygen-evolving Mn_4CaO_5 complex (Komenda et al., 2008; Nixon et al., 2010). Accessory factors, such as Ycf48, Psb27, and Psb28, associate transiently with PSII at specific stages of assembly but their functions remain unclear (Komenda et al., 2012b). PSII assembly in chloroplasts is likely to occur by a similar pathway, although additional accessory proteins appear to have been recruited (Chi et al., 2012; Nickelsen and Rengstl, 2013).

PSII is vulnerable to light-induced inactivation and is repaired via the selective replacement of the D1 protein, which is accompanied by a partial disassembly and reassembly of the complex (reviewed in Nixon et al., 2010). This process is especially important under high irradiance, which in cyanobacteria induces the synthesis of a group of single transmembrane helix proteins called Hlips (high-light-inducible proteins; Dolganov et al., 1995) or Scps (small CAB-like proteins; Funk and Vermaas, 1999) with sequence similarities to plant light-harvesting

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[W] Online version contains Web-only data.
www.plantcell.org/cgi/doi/10.1105/tpc.114.123919

complexes. Hlips have been detected in monomeric PSII and RC47 complexes (Promnares et al., 2006; Yao et al., 2007) and have been shown to prevent generation of singlet oxygen in PSII (Sinha et al., 2012) and to participate in regulation of chlorophyll biosynthesis (Xu et al., 2002; Hernandez-Prieto et al., 2011). Hlips contain a domain implicated in chlorophyll and carotenoid binding, and several studies have suggested binding of pigments to Hlips/Scps (Xu et al., 2004; Storm et al., 2008), but clear and convincing experimental evidence is still lacking.

In this study, we discovered that *Synechocystis* Ycf39, encoded by the *slr0399* gene, and two members of the Hlip family, HliC (ScpB) and HliD (ScpE), associate with the RCII complex. Our data demonstrate that Ycf39 and the two Hlips form a separate pigment-protein complex (designated the Ycf39-Hlip complex) containing chlorophyll and β-carotene, which is important for accumulation of the RC in vivo. Our data also suggest that the Ycf39-Hlip complex is involved in the delivery of recycled chlorophyll to newly synthesized D1 protein during the formation of the RCII subcomplex and protects newly synthesized D1 and RCII from light damage.

RESULTS

Identification of Ycf39 as a Component of a PSII RC Subcomplex

Our previous studies of PSII assembly in *Synechocystis* have revealed that strains unable to synthesize the inner antenna CP47 accumulate two RC assembly intermediate complexes designated RCII* and RCIIa (Sobotka et al., 2005; Dobáková et al., 2007; Komenda et al., 2008). These complexes differ in size on native gels, most probably due to the presence of additional component(s) in the larger RCII* complex. To explore this further, we separated solubilized membrane complexes isolated from the CP47-less strain (ΔCP47) by clear-native (CN) electrophoresis (Figure 1A). After separation by SDS-PAGE in the second dimension and staining of protein subunits, we used mass spectrometry (MS) to identify two well-stained proteins that comigrated with the RCII* complex. The larger one was the Ycf48 assembly factor found previously in RCII* (Komenda et al., 2008), while the smaller protein, of ~40 kD, was identified as Ycf39, the product of the *slr0399* gene (Figure 1A, 2D Sypro).

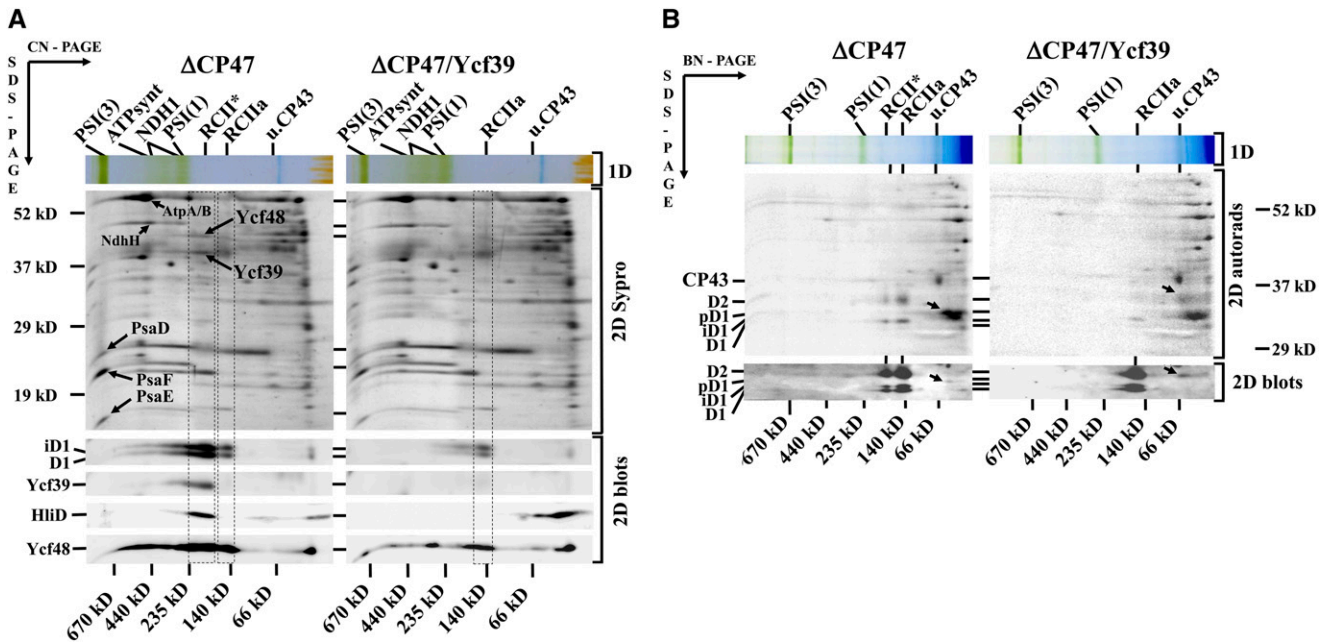

Figure 1. Identification of Ycf39 as a Component of the PSII RC Complex RCII* and Effect of Ycf39 on Protein Biosynthesis in a Strain Lacking the PSII Antenna CP47.

(A) Membranes isolated from cells of ΔCP47 and ΔCP47/Ycf39 deletion strains grown in the presence of Glc were analyzed by 2D-CN/SDS-PAGE. The first-dimension native gel was photographed (1D) and after SDS-PAGE in the 2nd dimension the gel was stained by Sypro (2D Sypro), and bands designated by arrows were identified by MS. Alternatively, the gel was blotted and probed with antibodies specific for D1, Ycf39, HliD, and Ycf48 (2D blots). The designation of complexes: PSI(3) and PSI(1), trimeric and monomeric PSI; ATPsynt, ATP synthase; NDH1, NADH dehydrogenase; RCII* and RCIIa, two PSII RC complexes lacking CP47 and CP43; u.CP43, unassembled CP43; iD1 and D1 indicate the partially and fully processed forms of the D1 protein, respectively. Each loaded sample contained 5 μg chlorophyll.

(B) Membranes isolated from cells of ΔCP47 and the ΔCP47/Ycf39 labeled by radioactive Met and Cys were analyzed by 2D-blue native (BN)/SDS-PAGE, the gel was blotted, the blot dried and exposed to a phosphor imager plate (2D autorads), and later used for detection of the D1 and D2 proteins by specific antibodies (2D blots). pD1 indicates the unprocessed form of D1, other designations are as in **(A)**. Unassembled pD1 (left panel) and D2 (right panel) are designated by oblique arrows.

The presence of Ycf39 in RCII* but not in the smaller RCIIa complex was also supported by immunoblotting experiments (Figure 1A, 2D blots). Surprisingly, a member of the Hlip family, HliD, was also present in RCII* but absent in RCIIa (Figure 1A, 2D blots), suggesting a link between Ycf39 and Hlips. To test the importance of Ycf39 for accumulation of RCII complexes, we deleted the *slr0399* gene in the ΔCP47 strain and membrane complexes of the resulting double mutant were subjected to analysis by 2D-CN/SDS-PAGE. The absence of RCII* in the ΔCP47/Ycf39 double mutant provided further confirmation that Ycf39 was an additional component of RCII*. Moreover, the overall level of RCII complexes markedly decreased (Figure 1A, 2D blots).

We also assessed the effect of deleting *ycf39* on the biosynthesis of membrane proteins in the ΔCP47 strain by radioactive protein labeling and subsequent 2D protein analysis (Figure 1B). The autoradiograms showed a significantly reduced incorporation of radiolabel into D1 in the double mutant, suggesting a limitation in the biosynthesis of D1 in the absence of Ycf39. This was further supported by immunoblot analysis of the 2D blot using antibodies specific for D1 and D2 (Figure 1B, 2D blots). While in the single ΔCP47 strain a small fraction of precursor D1 (pD1) is always observed in the region of unassembled proteins (see also Komenda et al., 2004), this fraction was missing in the double mutant and, instead, there was an increased level of unassembled D2 protein, shown previously to accumulate in the absence of D1 (Komenda et al., 2004).

Ycf39 Is Associated with the Cytoplasmic Surface of the Membrane

Ycf39 is predicted to contain an NAD(P)H binding domain and to be a soluble member of the short-chain alcohol dehydrogenase/reductase superfamily (Ermakova-Gerdes and Vermaas, 1999). Analysis of membrane and soluble fractions by immunoblotting revealed that Ycf39 was actually strongly associated with the membrane despite the lack of a predicted transmembrane region (Supplemental Figure 1B). Furthermore the sensitivity of Ycf39 to degradation by trypsin in right-side-out membrane vesicles (Komenda, 2000; Dobáková et al., 2009) and the lack of a predicted lumenal-targeting sequence suggested a location on the cytoplasmic side of the membrane.

Identification of a Chlorophyll Binding Complex Containing Ycf39, HliC, and HliD

To isolate complexes associated with Ycf39, we constructed a CP47-less strain expressing a 3xFlag-tagged version of the protein (Flag-Ycf39), which we purified under native conditions using a Flag-affinity column. The obtained eluate contained both chlorophyll and carotenoids as indicated by its absorption spectrum (Supplemental Figure 2A) and confirmed by HPLC analysis which demonstrated the presence of chlorophyll, β-carotene, and pheophytin (Supplemental Figure 3). Trace amounts of the carotenoids myxoxanthophyll and zeaxantin were detected but are likely to be impurities rather than regular components of the complexes. Moreover, the maximum of the 77K chlorophyll fluorescence spectrum at 682 nm suggested the isolated preparation was related to PSII as photosystem I (PSI) emits at ~720 to 730 nm under these conditions (Shen et al., 1993) (Supplemental Figure 2A).

Pigment proteins pulled down with Flag-Ycf39 were identified following 2D-CN/SDS-PAGE (Figure 2A). The analysis by native PAGE revealed the presence of four pigmented bands. The most intense yellow-green band corresponded to RCII* as revealed by its relative size (~200 kD) and polypeptide composition. Immunoblot and/or MS analysis (Supplemental Table 1) revealed the presence of D2, D1 (in its mature form as well as its incompletely processed form iD1), PsbE and PsbF of Cyt *b*-559, and PsbI. Furthermore, in the region around 45 kD, we detected Flag-Ycf39 and Ycf48. Finally, in the region around 5 to 7 kD, we also identified members of the Hlip family: HliD (ScpE) and HliC (ScpB).

A second smaller pigment-protein complex (of ~60 kD) contained Flag-Ycf39 together with HliD and HliC and represents a previously undiscovered pigment-protein complex of *Synechocystis*, which we designate the Ycf39-Hlip complex. A third pigmented band at the edge of the native gel most probably represented free pigments in detergent/lipid micelles, but there was also a distinct band of HliD, which might also bind pigment. We always detected a variable amount of another pigment-protein complex, migrating slightly faster than the RCII* complex. This complex contained most of the proteins detected in RCII* but did not contain Ycf39 and the Hlips, and its size corresponded to the size of RCIIa complex detected in thylakoids of the CP47-less strain. The lack of Ycf39 suggests that this complex results from fragmentation of the RCII* complex during purification or electrophoresis. Interestingly, this complex was highly fluorescent (Figure 2A, 1D LAS fluor), a feature that is usual for pigment-protein complexes related to PSII (Kopečná et al., 2013). However, both RCII* and the Flag-Ycf39-Hlip complex showed much lower fluorescence at room temperature, indicating efficient quenching of the absorbed light energy. Finally, the largest green nonfluorescent band was assigned as the trimeric PSI complex according to its size of ~750 kD, detection of the PsaD subunit (Figure 2A), and a 77K fluorescence emission band at 722 nm. However, the absence of a 722-nm chlorophyll fluorescence band at 77K in the pull-down preparation (Supplemental Figure 2) indicated that the trimeric PSI complex was only a minor component.

The use of the CN gel system allowed us to measure the absorption and low-temperature fluorescence spectra of the pigmented bands directly in the gel. Both Flag-Ycf39-Hlip and RCII* contained chlorophyll and carotenoids, with absorption maxima at around 675 and 450 to 530 nm, respectively. A small shoulder at ~543 nm in the absorption spectrum of the RCII* complex also indicated the presence of pheophytin (Figure 2B, arrow), which was also detected in the crude Flag-Ycf39 preparation by HPLC (Supplemental Figure 3). The spectrum of the Flag-Ycf39-Hlip complex was typified by a distinct absorption band at around 520 nm. Such a red shift in the β-carotene absorption band would suggest a special environment of this pigment within the complex (Figure 2B, left panel). The 77K chlorophyll fluorescence maximum at 681 nm was similar for both complexes (Figure 2B, right panel). Finally, the absorption maximum at 678 nm and 77K chlorophyll fluorescence band at ~722 nm confirmed the identity of the largest green band as PSI.

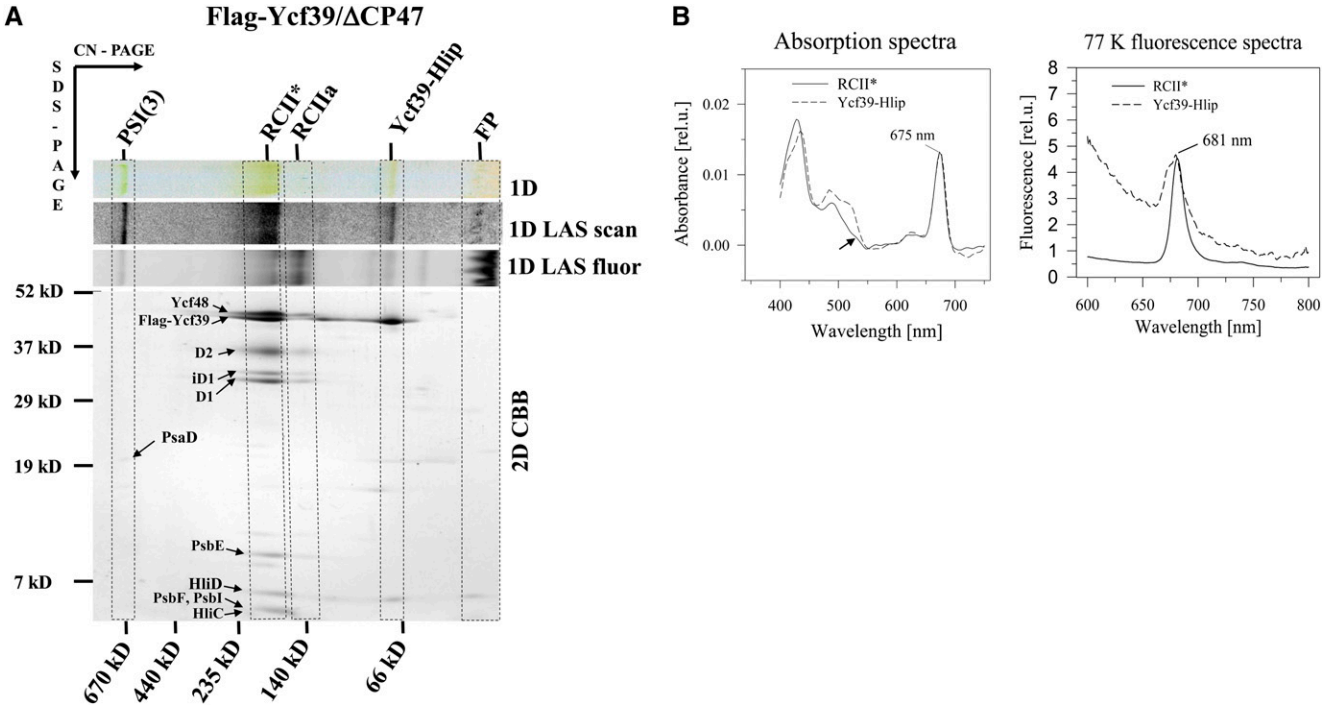

Figure 2. Analysis of the Flag-Ycf39 Preparation Using 2D-CN/SDS-PAGE and Room Temperature Absorption and 77K Chlorophyll Fluorescence Spectra of RCII* and Ycf39-Hlip Complexes in Gel.

(A) The preparation was isolated using anti-Flag affinity resin from membranes of the *Synechocystis* mutant expressing Flag-Ycf39 instead of Ycf39 and lacking CP47. It was subsequently analyzed by 2D-CN/SDS-PAGE. The first-dimension native gel was photographed (1D) or scanned by LAS 4000 for absorption (1D LAS scan) or fluorescence (1D LAS fluor) and after SDS-PAGE in the 2nd dimension, the gel was stained by Coomassie blue (2D CBB) and the designated spots were identified by MS (Supplemental Table 1). FP are free pigments, and other designations are as in Figure 1. Due to the high resistance of the PSI(3) band to SDS, most of its subunits are not released from it on 2D gels and are therefore hardly visible.
(B) The left panel shows room temperature absorption spectra, and the right panel the 77K chlorophyll fluorescence spectra of RCII* (solid lines) and Ycf39-Hlip complexes (dashed lines) in gel. The arrow in the RCII* absorption spectrum designates the shoulder at 543 nm belonging to pheophytin.

In order to prove that the attachment of Flag-Ycf39 with the RCII* complex was not an artifact related to the use of a mutant lacking CP47, we performed the pull-down experiment with a wild-type strain expressing Flag-tagged Ycf39. Although the yield of the preparation was much lower on a cell basis, the composition of the isolated preparation was similar to that obtained from the ΔCP47 strain (Figure 3A). We analyzed this preparation by 2D-CN/SDS-PAGE in combination with immunoblotting and detected Ycf48, D1, D2, HliC, HliD, and the PsbI proteins. As in the ΔCP47 strain, Ycf39, HliC, and HliD were present both in the RCII* complex and in a separate pigmented Ycf39-Hlip complex. In both preparations, we also detected a fluorescent band between the Ycf39-Hlip complex and free pigments. Based on the result of the immunoblot, it was ascribed to the D1-PsbI complex (Figure 3A, 1D LAS fluor) with its strong fluorescence suggesting the presence of bound chlorophyll.

HliC and HliD Are Needed for Pigment Binding and Stable Association of Ycf39 with PSII

To assess the importance of the Hlips for binding of Ycf39 to RCII, we constructed and analyzed CP47-less mutants lacking either HliC or HliD. The 2D electrophoresis confirmed that the absence of HliC, and especially HliD, led to a significant decrease in the abundance of RCII* (Supplemental Figure 4). We also constructed a CP47-less mutant expressing Flag-tagged Ycf39 but lacking HliD. The preparation isolated from this strain contained almost 10-fold less chlorophyll but lacked RCII and instead contained PSI as judged by absorption spectroscopy and 77K fluorescence emission spectroscopy (Supplemental Figure 2B). The preparation also lacked HliC, consistent with the previously identified close relationship between expression of HliC and HliD (Kufryk et al., 2008). Importantly, the Flag-tagged Ycf39 protein isolated from this strain did not bind detectable pigment following native gel electrophoresis (Figure 3B). This experiment showed that Hlips are required for binding of chlorophyll and β-carotene in the Ycf39-Hlip complex, while Ycf39 alone does not bind pigments.

The Ycf39-Hlip Complex Binds to D1 at an Early Stage in Assembly

To test whether the Ycf39-Hlip complex was able to bind to unassembled D1, we also expressed and isolated Flag-tagged Ycf39 from a strain lacking D2, which accumulates low levels of D1 but is unable to assemble RCII or larger PSII core complexes

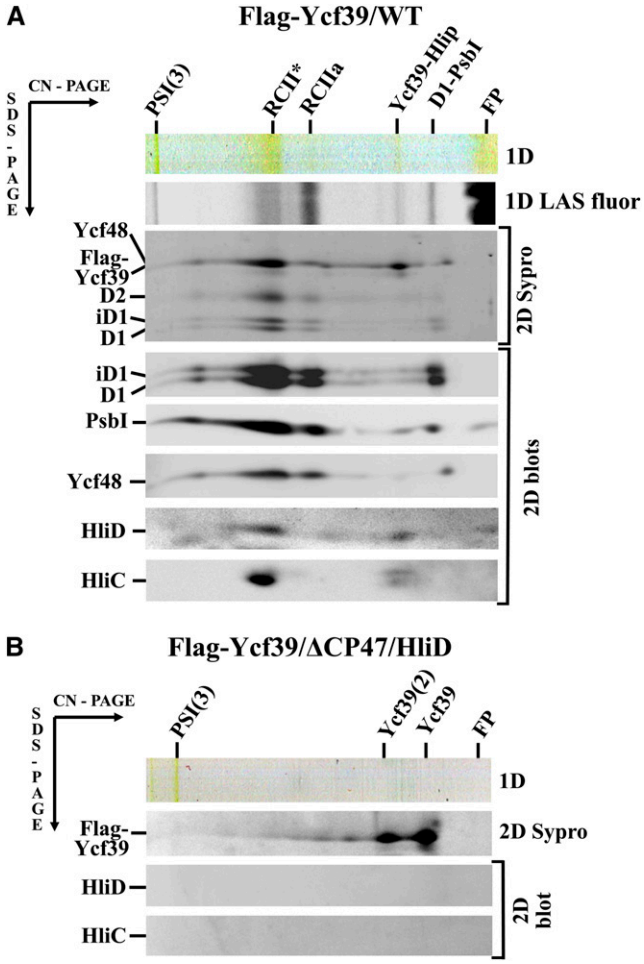

Figure 3. Pigment Proteins in Flag-Ycf39 Preparations from the Wild Type and a Strain Lacking CP47 and HliD.

The preparations from the wild type **(A)** and from the strain lacking CP47 and HliD **(B)**, both expressing Flag-Ycf39 instead of Ycf39, were isolated using anti-Flag affinity resin. Pigment proteins were subsequently separated by 2D-CN/SDS-PAGE. The first-dimension native gel was photographed (1D) and scanned by LAS 4000 for fluorescence (1D LAS fluor), after SDS-PAGE in the second dimension the gel was stained by Sypro orange (2D Sypro), electroblotted to polyvinylidene fluoride membrane, and probed with specific antibodies [2D blot(s)]. Designation of complexes is as in Figures 1 and 2, the Ycf39(2) is most probably the dimeric form of Ycf39, and D1-PsbI is a small fluorescent complex of (i) D1 and the small PSII subunit PsbI.

(Komenda et al., 2004). In the preparation we detected, in addition to Flag-Ycf39, D1 in the mature as well as incompletely processed form (iD1) together with Ycf48, HliC, and HliD (Figure 4A). Interestingly, the FtsH2/FtsH3 heterocomplex involved in D1 degradation during PSII repair was also present. By contrast, the Flag-Ycf39 preparation isolated from a D1-less strain, which is still able to accumulate low levels of the D2/Cyt b-559 subcomplex (Komenda et al., 2008), contained HliD, HliC but neither D2 nor Ycf48 (Figure 4B; Supplemental Table 1).

The DE Loop of D1 Is Important for Binding of Ycf39-Hlip Complex

The results thus far suggested that the D1 protein provides the binding site for the Ycf39-Hlip complex within RCII*. To locate this site more precisely, we tested two strains having either a 20–amino acid deletion at the N terminus (A20; Komenda et al., 2007) or a 25–amino acid deletion in the DE loop between the fourth and fifth transmembrane helices of D1 (R225-V249, PCD mutant; Mulo et al., 1997) for the presence of RCII* during radioactive labeling at low temperature, which leads to an increased accumulation of RCII subcomplexes (Komenda et al., 2008). The result showed the lack of RCII* accumulation in the PCD but not in the A20 mutant (Supplemental Figure 5), indicating a possible Ycf39 binding site in the R225-V249 segment of D1. During biosynthesis of the D1 protein, this segment is released from the ribosome and becomes available for Ycf39 binding when the fifth transmembrane helix is still buried within the ribosome. This moment corresponds well to a putative pause in biosynthesis of chloroplast D1, which is assumed to allow chlorophyll binding (Kim et al., 1991).

The preparations from both RCII-less strains and especially from the D1-less strain showed a significant content of 50S and 30S ribosomal subunits and, even more importantly, chlorophyll synthase (ChlG), which is the ultimate enzyme responsible for attachment of geranylgeranyl/phytyl group to Chlide during chlorophyll biosynthesis (Bollivar, 2006) (Figure 4B). Thus, the Ycf39-Hlip complex appears to associate with ribosomes and ChlG, implicating Ycf39 as playing a role at an early stage in D1 biosynthesis when chlorophyll is required for insertion into the growing polypeptide chain.

A Mutant Lacking Ycf39 Is More Sensitive to Photoinhibition

To gain more information on the physiological importance of Ycf39, we characterized a *ycf39* deletion strain and compared it with the wild type. In agreement with a previous report (Ermakova-Gerdes and Vermaas, 1999), there was no detectable difference in the phenotype between ΔYcf39 and the wild type in terms of growth rate, pigment composition, 77K chlorophyll fluorescence spectra, and rate of oxygen evolution at different irradiances. However, PSII in the ΔYcf39 strain was clearly more sensitive than in wild type to high irradiance as judged by the more rapid decline in the rate of oxygen evolution during the first 45 min of illumination at 700 μmol photons m^{-2} s^{-1} (Figure 5A). After this period, oxygen evolution stabilized in the mutant, which suggested that the photoprotective processes counteracting fast PSII photodamage are less efficient in the mutant during the initial period of high-light treatment but not at later points. Moreover, assessment of D1 protein turnover did not show any significant difference between the strains (Supplemental Figure 6). However, more detailed analysis by 2D gels revealed that the amount of labeled D1 protein was slightly lower in the PSII core complexes of the mutant (Figure 5B), and the mutant also showed a dramatic reduction in the level of labeled iD1 in the RCII complexes. Compared with the wild type, the mutant also showed increased accumulation of newly synthesized D1, D2, CP47, and CP43 in the unassembled protein region of the gel, consistent with a block in

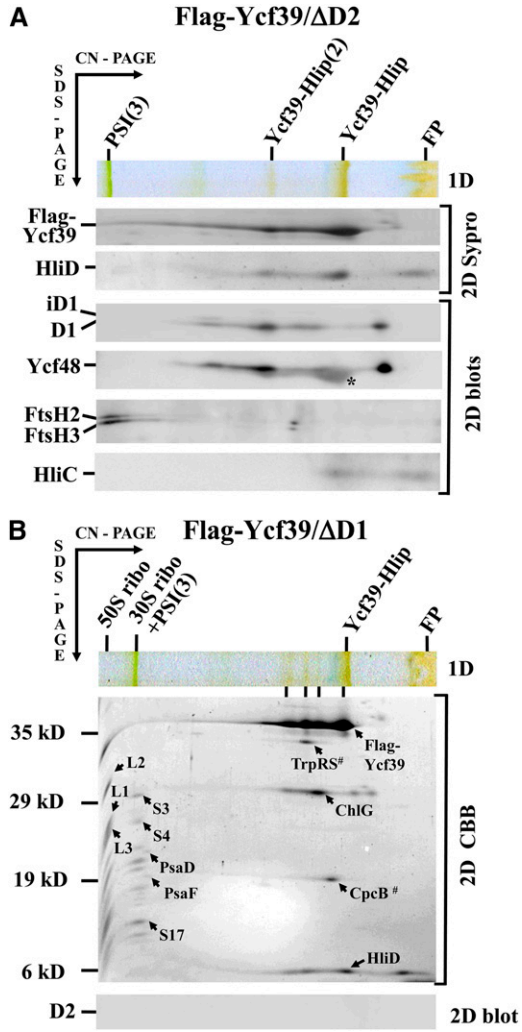

Figure 4. 2D-CN/SDS-PAGE Analysis of the Flag-Ycf39 Preparations from D2-Less and D1-Less Strains Expressing Flag-Ycf39.

The preparations from D2-less **(A)** and D1-less **(B)** strains expressing Flag-Ycf39 instead of Ycf39 were isolated using anti-Flag affinity resin. Pigment-proteins were subsequently separated by 2D-CN/SDS-PAGE. The first-dimension native gel was photographed (1D), and after SDS-PAGE in the second dimension, the gel in **(A)** was stained by Sypro Orange (2D Sypro), blotted onto polyvinylidene fluoride membrane, and D1, Ycf48, FtsH2, FtsH3, and HliC were detected by specific antibodies (2D blots). Asterisk indicates unspecific reaction of the flagged Ycf39 partly overlapping the signal of Ycf48. In **(B)**, the gel was stained with Coomassie blue (2D CBB) and selected proteins identified by MS. Complexes are designated as in Figure 3. 50S and 30S ribo designate large and small subunit of ribosomes, and other designations are as in Figure 2. The following proteins were identified in the preparation from the D1-less strain by MS: L1 (Rpl1, Sll1744), L2 (Rpl2, Sll1260), and L3 (Rpl3, Sll1799) of the large 50S ribosomal subunit subunit; S3 (Rps3, Sll1804), S4 (Rps4, Slr0469), and S17 (Rps17, Ssl3437) of the small ribosomal subunit; Flagged Ycf39 (Slr0399); ChlG (chlorophyll synthase, Slr0056); HliD (ScpE, Ssr1789). The bands with hashtag, CpcB (Sll157) and TrpRS (tryptophanyl-tRNA-synthetase, Slr1884), are standard unspecific interactors of the Flag resin. The 2D blot indicates the lack of the D2 protein in the preparation.

the formation of RCII and larger complexes. Overall, these data suggest that the Ycf39-Hlip complex is important for maintaining PSII activity upon sudden exposure to high irradiances at the level of D1 incorporation into RCII complexes.

Ycf39 Is Needed for Efficient Chlorophyll Recycling

Association of Ycf39 with D1, Hlips, ChlG, and ribosomes implies the possible involvement of Ycf39 in the insertion of chlorophyll into newly synthesized D1, either during de novo assembly or PSII repair. To investigate this further, we analyzed differences in the level of pigments and chlorophyll precursors and their changes induced by exposure of the wild-type and Ycf39-less cells to high light. No significant differences were observed between the strains under the experimental conditions used and a similar result was also obtained for the strains lacking CP47. However, since the role of Ycf39 seems to be restricted to the biosynthesis of the D1 protein, which contains only a small fraction of total cellular chlorophyll present in wild-type cells, specific changes related just to the D1 protein could be masked by the insertion of chlorophyll into other chlorophyll binding proteins. Therefore, we constructed a mutant that contains the D1 and D2 proteins but lacks PSI, CP47, and CP43, together with its variant lacking Ycf39. We exposed these mutants to a short high-light treatment in the presence of the chlorophyll biosynthesis inhibitor gabaculine (Guikema et al., 1986) and simultaneously monitored the levels of the D1 protein and early chlorophyll precursors Mg-protoporphyrin and its monomethyl ester. The ΔPSI/CP47/CP43/Ycf39 strain showed a strong depletion of Mg protoporphyrin during 30-min exposure to high light indicating consumption of chlorophyll biosynthesis intermediates for biosynthesis of new chlorophyll while the precursors in the control strain remained stable (Figure 6A). Immunoblots showed a stable content of both iD1 and D1 proteins during light treatment in the control strain, while the level of both proteins decreased by ~30% after light treatment of the Ycf39-less strain (Figure 6B). These data suggest a block in the reuse of chlorophyll and a higher requirement of newly synthesized chlorophyll for de novo biosynthesis of the D1 protein in the absence of Ycf39.

DISCUSSION

Despite its obvious physiological importance, little is known about how chlorophyll is delivered to the apo-polypeptides during assembly and repair of the photosynthetic apparatus in cyanobacteria and chloroplasts. Here, we provide evidence that the Ycf39-Hlip pigment-protein complex is important for chlorophyll delivery to the D1 subunit during the initial stages of PSII assembly in *Synechocystis*.

Ycf39 was identified following analysis of the polypeptide composition of the RCII* complex, an early assembly intermediate of PSII lacking the inner antennae CP47 and CP43 (Figure 1). Isolation of Flag-tagged Ycf39 revealed that this protein forms a chlorophyll and β-carotene binding protein complex with two members of the Hlip family, HliC and HliD. In the wild type, the complex was mostly present in its free form due to the low content of RCII subcomplexes (Supplemental

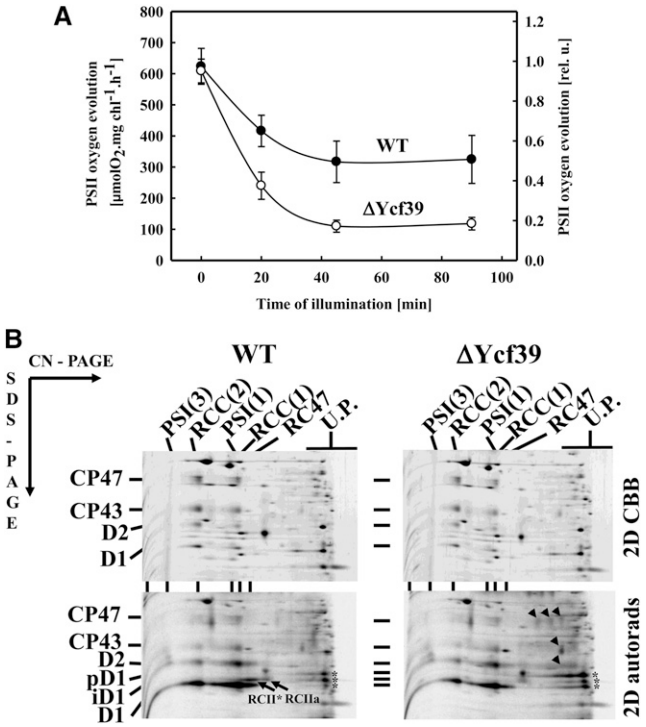

Figure 5. PSII Repair and Biosynthesis of PSII Proteins in Wild-Type and ΔYcf39 Strains under High Irradiance.

(A) Wild-type cells (closed symbols) and ΔYcf39 (empty symbols) were illuminated with 700 μmol photons m^{-2} s^{-1} of white light for 90 min, and during illumination, PSII oxygen-evolving activity was assayed in whole cells to examine PSII repair. The initial values of oxygen evolution were 624 ± 58 and 609 ± 38 μmol O_2 mg chlorophyll^{-1} h^{-1} for the wild type and ΔYcf39, respectively (values are means of six measurements ± SD from three independent experiments).

(B) Membranes isolated from radioactively labeled cells were analyzed by 2D-CN/SDS-PAGE. The gels were stained by Coomassie blue (2D CBB) and exposed to a phosphor imager plate (2D autorads). Designation of complexes: RCC(2) and RCC(1), dimeric and monomeric PSII core complexes; RC47, PSII core complex lacking CP43; U.P., unassembled proteins; other designations are as in Figure 1. Asterisks indicate unassembled forms of unprocessed, partially processed, and processed D1 protein (their identity was confirmed by immunodetection), arrows indicate RCII complexes, which are almost missing in the mutant; arrowheads designate unassembled CP47, CP43, and D2 waiting for the D1 protein. Each loaded sample contained 5 μg chlorophyll.

Figure 4), but in the strain lacking CP47 and therefore unable to form larger PSII core complexes, a significant portion of Ycf39-Hlip was found attached to the RCIIa subcomplex forming the larger subcomplex RCII* (Figures 1 and 2). The RCII* subcomplex contains the previously identified PSII subunits: Mature D1 and its incompletely processed form iD1, D2, both subunits of Cyt *b*-559 (PsbE and PsbF), and PsbI (Komenda et al., 2004, 2008; Dobáková et al., 2007). Occasionally, the PsbY subunit could also be detected in the complex (Supplemental Table 1). The subunit composition of the RCII* complex corresponds well to the latest PSII structural models (Ferreira et al., 2004; Guskov et al., 2009; Umena et al., 2011) since the presence of most other small subunits and lumenal PSII proteins is dependent on the antenna chlorophyll-proteins CP47 and CP43. These two chlorophyll-proteins attach to the RCII complex as separate modules, together with neighboring small subunits and pigments, later in the process of PSII assembly (Boehm et al., 2011; Komenda et al., 2012a). In agreement with a previous report (Komenda et al., 2008), RCII* also contained the Ycf48 assembly factor (Figure 1A). Several minor protein bands that we did not succeed in identifying due to their low abundance, were also present so the existence of other substoichiometric accessory factors in RCII* remains possible.

The low abundance of the RCII complexes in vivo has until now prevented their isolation and characterization. The absorption spectrum of the RCII* separated by native gel electrophoresis indicated the presence of a higher number of chlorophyll and β-carotene molecules in comparison with the RCII complex previously isolated from *Synechocystis* by stripping off the inner PSII antennae from the PSII core (Tomo et al., 2008), which is consistent with additional binding of pigments to the Ycf39-Hlip complex. Moreover, a small shoulder at ~545 nm is consistent with the presence of pheophytin. There are two pheophytin molecules in the D1/D2 complex isolated by fragmentation of larger core complexes, one of which is used as the primary electron acceptor (Nanba and Satoh, 1987). So, it is possible that during PSII assembly, the pigments needed for primary charge separation are already present in the RCII assembly intermediates.

Separation of the Ycf39-Hlip complex from other complexes and proteins by native gel electrophoresis and measurement of its absorption spectra also provided the strongest support yet for chlorophyll and β-carotene binding to Hlips. When we isolated Flag-Ycf39 from the strain lacking both CP47 and HliD, no pigmented band appeared in the low molecular weight region of the gel despite the large amount of the Flag-Ycf39 present (Figure 3B). This is consistent with bioinformatic analyses of Ycf39 indicating it to be a member of the short-chain alcohol dehydrogenase/reductase family unrelated to pigment binding.

The Ycf39 protein overaccumulated in several mutants unable to form either PSII core or RCII complexes due to the lack of D1, D2, or CP47. Immunoblot analysis showed increased amounts of Ycf39 in all three of the tested PSII mutants with its amount agreeing well with the increased content of HliD (and carotenoids) (Supplemental Figure 7A). Amounts of Ycf39 and HliD also increased in parallel in wild-type cells exposed to high light (Supplemental Figure 7B). Interestingly, using 2D gel analysis, we detected much higher levels of two other members of the Hli family, HliA/B, bound to the PSII core complex of the Ycf39-less strain in comparison with the wild-type strain (Supplemental Figure 8). Accumulation of HliA/B could represent a possible compensatory mechanism induced in the absence of Ycf39.

Flag-Ycf39-Hlip was the main complex isolated from strains lacking either the D1 or D2 proteins (Figure 4), but in these strains, it was copurified with other protein components. Purification of the complex from the strain expressing Flag-tagged Ycf39 and lacking D2 revealed association of the complex with the D1 protein together with PsbI and Ycf48, confirming the involvement of these two latter proteins in the very early stage of D1 biosynthesis (Dobáková et al., 2007; Komenda et al., 2008).

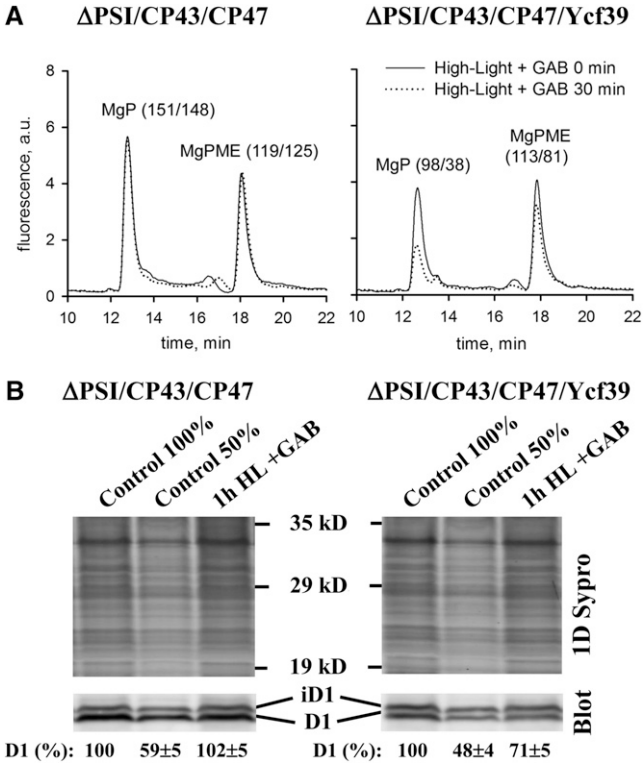

Figure 6. Analysis of Early Chlorophyll Precursors and the D1 Protein Level in Cells of ΔPSI/CP47/CP43 and ΔPSI/CP47/CP43/Ycf39 Strains after Exposure to 300 μmol Photons m^{-2} s^{-1}.

(A) Chromatograms of chlorophyll precursors separated by HPLC from cells of both strains after 30-min dark incubation with 20 μM gabaculine (GAB 0 min) and after an additional 30-min high-light treatment (GAB 30 min).

(B) Membrane proteins from control and cells treated with high light for 60 min in the presence of gabaculine (1 h HL + GAB) were analyzed by SDS-PAGE, the gels were electroblotted to polyvinylidene fluoride membrane and probed with antibodies specific for D1 (blot). Loading of the gel was checked by staining with Sypro Orange (1D Sypro). Chlorophyll (0.2 and 0.1 μg) was loaded for control 100 and 50%, respectively, 0.2 μg for the Ycf39-less mutant. The values represent mean ± SD from three measurements.

The binding of Ycf39 to D1 may occur during a pause in translation of D1, possibly related to insertion of chlorophyll into D1 (Kim et al., 1991). By contrast, the main binding partners of the complex in the strain lacking D1 were ribosomes (namely the large ribosomal 50S subunit) and the enzyme chlorophyll synthase. Indeed, purified chlorophyll synthase has been recently shown to contain small amounts of Ycf39 (Chidgey et al., 2014). Based on these data, we hypothesize that the Ycf39-Hlip complex is present in a complex that participates in the coordinated delivery of chlorophyll to newly synthesized D1 protein. Free chlorophyll is known to be highly toxic in the membrane due to its ability to produce reactive oxygen species in the light (Apel and Hirt, 2004), and its biosynthesis and delivery must be carefully controlled. However, the almost identical phenotype of the wild type and ΔYcf39 under standard growth conditions is not consistent with the essential role of Ycf39 in this process. Given its crucial physiological importance, chlorophyll delivery to the D1 protein may occur via multiple pathways with some not involving Ycf39 as an essential component. Vermaas and colleagues have shown that most chlorophylls are actually recycled via Chlide during PSII repair (Vavilin and Vermaas, 2007). Based on our data (Figure 6), we hypothesize that the Ycf39-Hlip complex plays an important role in the reuse of chlorophyll released from degraded proteins for biosynthesis of D1.

According to our current working model, damaged D1 is degraded in *Synechocystis* by the FtsH2/FtsH3 heterohexameric protease complex (Boehm et al., 2012b); indeed, this complex was identified in the Ycf39 pull-down from the strain lacking D2 (Figure 4B). The released chlorophylls might then be immediately reused with the help of Hlips (Vavilin et al., 2007) via reactions involving detachment of the phytol tail (catalyzed by unknown enzyme) and its reattachment (Vavilin and Vermaas, 2007) catalyzed most probably by chlorophyll synthase. After association of Ycf39, Hlips, and chlorophyll synthase, the D1 protein emerging from membrane-bound ribosomes will be loaded with the reused chlorophylls and finally bound to the D2 protein to form RCII* (Figure 7). After attachment of the CP47 module (Komenda et al., 2012a), the Ycf39-Hlip complex is detached from the resulting RC47 complex (Boehm et al., 2012a) and used for biosynthesis and assembly of other D1 modules. The sensitivity of PSII activity in the *ycf39* null mutant to high light (Figure 5A) suggests that the use of recycled chlorophyll for D1 biosynthesis might be especially important at the initial stages of high light stress when de novo biosynthesis of chlorophyll is not yet fully induced. Upon onset of high light, PSII damage can be so fast that the biosynthesis of D1 is not fast enough to quickly replace D1 at the level of the RC47 complex and RCII complexes synthesized with the help of recycled chlorophyll might represent an alternative mode of repair in which either D1 or both D1 and D2 can be replaced.

A minor component of all isolated Flag-Ycf39 preparations was trimeric PSI, which is the most abundant complex of *Synechocystis* thylakoids. This complex was also present in the negative control eluate obtained after passing solubilized

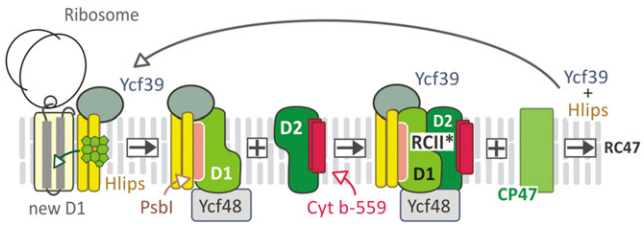

Figure 7. The Hypothetical Scheme for the Role of Ycf39-Hlip Complex in the Biosynthesis of D1 and Assembly of the RCII Complex.

Recycled chlorophylls are inserted via Ycf39-Hlip into D1 as it is synthesized and inserted into the membrane at a translocon-associated ribosome. D1 is then released and associates with PsbI and Ycf48 to form a D1 module, which binds to the D2 module, containing D2 and both subunits of cytochrome b-559, to form the RCII* assembly complex. Subsequent attachment of CP47, forming the RC47 complex, releases the Ycf39-Hlip complex for reuse.

thylakoids of the wild type through the anti-Flag affinity resin and therefore is possibly a contaminant. However, the level of trimer in isolated Ycf39-containing eluates was higher than in the control, so we do not yet exclude the possibility that Ycf39 binds to PSI and is involved in the release of chlorophyll from PSI for insertion into other pigment binding proteins, such as the D1 protein, as suggested recently (Kopečná et al., 2012).

The function of a NAD(P)H binding motif near the N terminus of Ycf39 remains elusive. Link et al. (2012) have pointed out that this motif could in fact bind RNA instead of being involved in redox reactions typical for the family of atypical short-chain dehydrogenases/reductases to which Ycf39 can be assigned (Kavanagh et al., 2008). As RNAs are components of ribosomes, the motif might represent a binding site for ribosomes or possibly for mRNA.

The strong quenching of chlorophyll fluorescence in RCII* but not in RCIIa also provides evidence for a role of the Ycf39-Hlip complex in safely dissipating excess excitation energy in the RCII* complex through nonphotochemical quenching, thereby protecting the RCII complex from light damage. We hypothesize that β-carotene bound to the Ycf39-Hlip complex may directly or indirectly via its own chlorophylls quench excitation energy from chlorophylls bound to the D1 protein. Although the orange carotenoid protein is known to play a photoprotective role in cyanobacteria (Kirilovsky and Kerfeld, 2012), it acts by binding to the phycobilisome light-harvesting complex, which is not able to bind to PSII assembly complexes (Wilson et al., 2006). The Ycf39-Hlip complex may also temporarily bind chlorophylls released from degraded RCII when the biosynthesis of new D1 is not yet completed. In both cases, formation of triplet chlorophylls and reactive singlet oxygen will be prevented (Sinha et al., 2012), thereby preventing damage to D1 (Figure 6B).

Ycf39 has previously been suggested to act as a chaperone helping to insert quinone into mutated Q_A binding pockets of PSII (Ermakova-Gerdes and Vermaas, 1999). However, Ycf39 is not actually needed for Q_A insertion into wild-type PSII (Ermakova-Gerdes and Vermaas, 1999), so it seems more likely that binding of Ycf39 protein on the cytoplasmic surface of RCII might have indirect effects on the access/binding of quinone to the Q_A binding pocket.

Hlips and Ycf39 have homologs in *Arabidopsis thaliana*, which suggests that these proteins might play a similar role in chloroplasts. Genes for one-helix-proteins (Ohps) homologous to Hlips have been identified in the *Arabidopsis* genome (Engelken et al., 2010) and like Hlips are also induced by high irradiance (Jansson et al., 2000; Andersson et al., 2003). The Ycf39 homolog from *Arabidopsis* called High Chlorophyll Fluorescence 244 (HCF244) (Link et al., 2012) is essential for normal accumulation of PSII and the protein has been shown to be needed for biosynthesis of the D1 protein, although the mechanism of its action has not been explained. Our data are in accordance with the close relationship between Ycf39 and biosynthesis of the D1 protein. It is possible that in *Arabidopsis* the absence of HCF244 does not allow de novo biosynthesis of the D1 protein, which would agree with the observed phenotype of the *hcf244* null mutant. However, in *Synechocystis* D1, biosynthesis is not completely blocked in the *ycf39* mutant and the block seems to mainly concern just a small fraction of the D1 protein destined for the assembly of new RCII complexes. On the other hand, most of the protein used for replacement of the damaged D1 protein appears to be synthesized normally as in the wild type (in agreement with this, no apparent difference in the *psbA* transcript level was found between the wild type and ΔYcf39); consequently, the phenotypes of the wild type and the ΔYcf39 mutant are very similar. This contrasts with the situation observed in *Arabidopsis* in which the absence of HCF244 leads to the almost complete loss of PSII accumulation in the mutant. This comparison again emphasizes the more drastic effect of a similar mutation in chloroplasts in comparison with cyanobacteria. It is possible that during de novo assembly of PSII in the chloroplast the newly synthesized D1 protein lacking bound HCF244 is efficiently recognized and degraded by proteases such as FtsH, while D1 in *Synechocystis* is able to escape the quality control system to some degree to allow formation of new PSII complexes.

METHODS

Construction and Cultivation of Cyanobacterial Strains

The previously constructed strains used in this study were derived from the Glc-tolerant strain of *Synechocystis* sp PCC 6803 (Williams, 1988) referred to as the wild type. The following previously described strains of the Glc-tolerant *Synechocystis* sp PCC 6803 strain were used: (1) the CP47 deletion strain (ΔCP47), in which the *psbB* gene is replaced by a spectinomycin resistance cassette (Eaton-Rye and Vermaas, 1991); (2) the *psbA* triple deletion strain (ΔD1) with cassette-less deletions in *psbA1* and *psbA3* genes and the *psbA2* gene replaced by a chloramphenicol resistance cassette (Nagarajan et al., 2011); (3) the *psbDIC*/*psbDII* deletion strain (ΔD2) with the *psbDIC* and *psbDII* genes encoding the D2 protein replaced by a chloramphenicol and a spectinomycin resistance cassette, respectively (Vermaas et al., 1990), and the *psaAB* deletion strain (ΔPSI) in which *psaAB* operon was replaced with a chloramphenicol resistance cassette (Shen et al., 1993). The ΔPSI strain was used to prepare the *psaAB*/*psbB*/*psbC* triple deletion strain (ΔPSI/CP47/CP43) in which *psbB* was additionally replaced by a spectinomycin resistance cassette and *psbC* interrupted by a kanamycin resistance cassette. The Hlip-lacking strains were transformed using genomic DNAs isolated from strains lacking HliC and HliD (Xu et al., 2002).

The transformation vector used to disrupt the *slr0399* gene encoding Ycf39 was constructed in two steps. First, *slr0399* along with 445 bp of upstream and 555 bp of downstream sequences were amplified from wild-type genomic DNA with primer set Slr0399-F (5′-GCCCCAGGACACG-GATTTTACCGTCAA-3′) and Slr0399-R (5′-GTTCAGGGAGAGCGCTTCC-CATAAAAGGTT-3′). The resulting fragments were then cloned into the multiple cloning region of pGEM-T Easy vector (Promega) to yield the initial vector pSlr0399 (Supplemental Figure 9A). Further modifications on pSlr0399 was then performed by replacing the open reading frame for Ycf39 between the *Eco*NI and *Hpa*I sites with an erythromycin resistance cassette via restriction digestion and ligation to create the final transformation vector termed pSlr0399ErmA (Supplemental Figure 9A). Sequencing confirmed that the orientation of the resistance cassette was the same as the orientation of *slr0399*. The pSlr0399ErmA plasmid was then used to transform the wild-type cells. Transformants were selected for erythromycin resistance and PCR was used to show integration of the selectable marker and elimination of the wild-type *slr0399* gene copy (Supplemental Figure 9B). The pSlr0399ErmA plasmid was also used for the transformation of other mutants like ΔD1, ΔD2, and ΔCP47 to create their Ycf39-less variants and their complete segregation was again checked by PCR (Supplemental Figure 9B).

Plasmid pPsbAIpetJ-Flag was used for construction of the strains expressing Flag-tagged Ycf39 under the control of the copper-regulated *petJ* promoter at the *psbAI* locus. The plasmid was constructed as follows: a 600-bp XbaI-Cfr9I fragment upstream and a 600-bp BglII-XmaJI fragment downstream of the *psbAI* gene were amplified by PCR and cloned into pETBlue-2 plasmid (Novagen). The *petJ* promoter from *Synechocystis* sp PCC 6803 (positions 846,614 to 846,331 according to CyanoBase) and 3xFlag coding sequence (Sigma-Aldrich) were amplified by PCR, ligated, and again amplified by PCR for cloning between the *psbAI* fragments. Finally, the kanamycin resistance gene (aphX) from pUC4K, amplified as a BamHI-*Bgl*II fragment, was cloned into the *Bgl*II site upstream of the second *psbAI* fragment leaving a single *Bgl*II site for cloning of *slr0399*. To clone the *slr0399* into the Flag plasmid, the *slr0399* gene was amplified by PCR using the following primers: NotI+*slr0399*-Fw, 5′-ATTAAAGCGGCCGCACGAGTTTTGGTGGTAGGCGG-3′, and *slr0399*+BglII-Rev, 5′-CAAACTAGTAGATCTAGAAAAAGAA-3′. After *Not*I and *Bgl*II digestion and ligation into the plasmid, the construct was used for the transformation of ΔSlr0399 deletion mutants. Mutants were selected for kanamycin resistance and were examined for segregation in the *psbAI* locus by PCR using gene-specific primers. To induce expression of the Flag-tagged Ycf39 protein, the strains were cultivated in BG-11 medium lacking $CuSO_4$.

The strains were grown in BG-11 as described previously (Kopečná et al., 2012). Growth media of nonautotrophically growing mutants were supplemented with 5 mM Glc. For gabaculine experiments, the cells of the strain ΔPSI/CP47/CP43 and its Ycf39-less variant were incubated with 20 μM gabaculine in the dark for 30 min, then the control samples for precursor assessment and immunoblot analysis were taken and the cells were subsequently exposed to irradiance of 300 μmol photons m^{-2} s^{-1} for additional 30 and 60 min, after which other samples were taken and analyzed. All experiments and measurements with cells were performed at least in triplicate and typical results are shown in figures.

Radioactive Labeling

Radioactive pulse (for 2D analysis) and pulse-chase (for 1D analysis) labeling of the cells was performed using a mixture of [^{35}S]Met and [^{35}S]Cys (Trans-label; MP Biochemicals) as described previously (Dobáková et al., 2009).

Preparation of Cellular Fractions and Isolation of Flag-Ycf39

Membranes were prepared by breaking the cells with zirkonia/silica beads according to the procedure described by Komenda and Barber (1995) and Dobáková et al. (2009). Flag-tagged Ycf39 was isolated using the anti-Flag M2 affinity gel (Sigma-Aldrich) after solubilization of membranes in 1.5% (w/v) *n*-dodecyl-β-D-maltoside.

Analysis of Proteins and Their Complexes

The membrane protein complexes were analyzed by blue-native or CN electrophoresis in combination with SDS-PAGE and immunoblotting as described (Komenda et al., 2012b). The previously described primary antibodies against the following proteins were used in this study: D1, D2, CP47, CP43, PsbI (Dobáková et al., 2007), and FtsH2 and FtsH3 (Boehm et al., 2012b). Moreover, we also used commercial anti-HliA/B and HliD (Agrisera), anti-Flag (Sigma-Aldrich) and our own antibodies against Ycf48, Ycf39, and HliC. The antiserum to *Synechocystis* Ycf48 (Slr2034) was raised against residues 37 to 342 expressed in *Escherichia coli* as an N-terminal His-tagged fusion protein and isolated by immobilized nickel affinity chromatography as described by Michoux et al. (2010). It was used as a crude antiserum at a dilution of 5000. Antibodies against Ycf39 (Slr0399) and HliC (Ssl1633) were raised in rabbit against a conjugate of keyhole limpet hemocyanin with a peptide 311-322 of Ycf39 and 2-17 of HliC, respectively. Antisera were purified using the corresponding immobilized peptides and used at a dilution of 2000 for anti Ycf39 and 500 for anti-HliC.

Tryptic in-Gel Digestion and Protein Identification by MS

The initial identification of Ycf39 in the Coomassie Brilliant Blue–stained protein spot separated by 2D-CN/SDS-PAGE from the CP47-less strain was performed by the Ultraflex III MALDI-TOF/TOF instrument (Bruker Daltonics) as described (Komenda et al., 2012b); all other MS analyses were done on a NanoAcquity UPLC (Waters) online coupled to an ESI Q-ToF Premier mass spectrometer (Waters) as described (Janouškovec et al., 2013).

Pigment Analyses, Spectroscopy, and Polarography Methods

Chlorophyll concentration was measured in 100% methanol according to Wellburn (1994), and quantitative determination of pigments in isolated preparations was performed by HPLC as described (Kopečná et al., 2012). For quantitative determination of chlorophyll precursors, a 3-mL culture at OD_{750} = 0.5 to 0.6 was spun down and resuspended in 20 μL water. Pigments were extracted with an excess of 70% methanol/30% water, filtered, and immediately injected onto an HPLC (Agilent-1200). Separation was performed on a reverse phase column (ReproSil pur 100, C8, 3-μm particle size, 4 × 150 mm; Watrex) with 35% methanol and 15% acetonitrile in 0.25 M pyridine (solvent A) and 50% methanol in acetonitrile as solvents B. Pigments were eluted with a gradient of solvent B (40 to 52% in 5 min) followed by 52 to 55% of solvent B in 30 min at a flow rate of 0.8 mL min^{-1} at 40°C. Eluted pigments were detected by a fluorescence detector set at 416/595 nm to detect Mg-protoporphyrin and Mg-protoporphyrin methylester. Chlorophyll fluorescence emission spectra at 77K were measured using an Aminco Bowman Series 2 luminescence spectrometer (Spectronic Unicam) as described (Sinha et al., 2012). Absorption spectra were measured using a Shimadzu UV3000 spectrophotometer, and the light-saturated steady state rate of oxygen evolution was assessed polarographically in the presence of artificial electron acceptors as described (Komenda et al., 2007).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: Ycf39 (Slr0399), NP_441851; HliC (Ssl1633), NP_440923; and HliD (Ssr1789), NP_440269.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Localization of Ycf39 in the Cells of *Synechocystis*.

Supplemental Figure 2. Room Temperature Absorption and 77K Chlorophyll Fluorescence Spectra of Flag-Tagged Ycf39 Preparations Isolated from the *Synechocystis* CP47-Less Mutant Expressing Flag-Ycf39 and Containing or Lacking HliD.

Supplemental Figure 3. HPLC Analysis of Pigments Present in the Flag-Tagged Ycf39 Preparation Isolated from the *Synechocystis* CP47-Less Mutant Expressing Flag-Ycf39 Instead of Ycf39.

Supplemental Figure 4. 2D Analysis of Membrane Protein Complexes of the Wild Type, ΔCP47, and ΔCP47 Lacking HliC or HliD Proteins.

Supplemental Figure 5. Biosynthesis of the PSII Proteins in Strains with N-Terminal Deletion (A20) and Deletion in the DE Loop of D1 [Δ(R225-V249)] under High Irradiance.

Supplemental Figure 6. The Rate of the D1 Degradation under High Irradiance Is Not Changed upon the Deletion of Ycf39.

Supplemental Figure 7. Levels of Ycf39 and HliD in Various PSII Mutants Unable to Assemble PSII Core Complexes and in Wild Type Exposed to High Irradiance.

Supplemental Figure 8. 2D Analysis of Membranes from Cells of the Wild Type and ΔYcf39 Deletion Strain before and after Their Treatment under High Irradiance of 500 μmol Photons m^{-2} s^{-1}.

Supplemental Figure 9. Construction and Verification of the *slr0399* Deletion.

Supplemental Table 1. PSII-Related Proteins Identified by MS and Immunoblotting in the RCII* Complex Separated by 2D-CN/SDS PAGE from the Flag-Ycf39 Preparation Isolated from the ΔCP47 Strain.

Supplemental Table 2. List of PSII- and Chlorophyll Biosynthesis-Related Proteins Identified in Preparations of Flag-Ycf39 Isolated from Wild-Type, HliD-, CP47-, D1-, and D2-Less Strains Expressing Flag-Ycf39.

ACKNOWLEDGMENTS

We thank Jan Pilný, Lenka Moravcová, and Vendula Krynická for excellent technical assistance during protein, pigment, and transcript analyses. J. Knoppová, J. Komenda, R.S., and M.T. were supported by projects Algatech (CZ.1.05/2.1.00/03.0110), RVO61388971, and P501/11/0377 of the Grant Agency of the Czech Republic; P.H. by project "Prague Infrastructure for Structure Biology and Metabolomics" (CZ.2.16/3.1.00/24023); and J.Y. and P.J.N. were supported by the UK Biotechnology and Biological Sciences Research Council (Grants BB/F020554/1 and BB/L003260/1).

AUTHOR CONTRIBUTIONS

J. Knoppová, R.S., P.J.N., and J. Komenda designed research. J. Knoppová, R.S., M.T., J.Y., P.K., P.H., and J. Komenda performed research. J. Knoppová, R.S., P.K., P.H., P.J.N., and J. Komenda analyzed the data. J. Knoppová, R.S., P.J.N., and J. Komenda wrote the article.

Received February 5, 2014; revised February 25, 2014; accepted March 7, 2014; published March 28, 2014.

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