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

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The role of the slr2034 (ycf48) gene product in the assembly and repair of photosystem II (PSII) has been studied in the cyanobacterium Synechocystis PCC 6803. YCF48 (HCF136) is involved in the assembly of Arabidopsis thaliana PSII reaction center (RC) complexes but its mode of action is unclear. We show here that YCF48 is a component of two cyanobacterial PSII RC-like complexes but its mode of action is unclear. We show here that YCF48 is a component of two cyanobacterial PSII RC-like complexes in vivo but is absent in larger PSII core complexes. Interruption of ycf48 slowed the formation of PSII complexes in wild type, as judged from pulse-labeling experiments, and caused a decrease in the final level of PSII core complexes in wild type and a marked reduction in the levels of PSII assembly complexes in strains lacking either CP43 or CP47. Absence of YCF48 also led to a dramatic decrease in the levels of the COOH-terminal precursor (pD1) and the partially processed form, iD1, in a variety of PSII mutants and only low levels of unassembled mature D1 were observed. Yeast two-hybrid analyses using the split ubiquitin system showed an interaction of YCF48 with unassembled pD1 and, to a lesser extent, unassembled iD1, but not with unassembled mature D1 or D2. Overall our results indicate a role for YCF48 in the stabilization of newly synthesized pD1 and in its subsequent binding to a D2-cytochrome b559 pre-complex, also identified in this study. Besides a role in assembly, we show for the first time that YCF48 also functions in the selective replacement of photodamaged D1 during PSII repair.

The photosystem II (PSII)2 complex of plants, algae, and cyanobacteria is a multisubunit pigment–protein complex responsible for water oxidation during oxygenic photosynthesis (1). The complex consists of over 20 intrinsic and peripheral membrane proteins, which must be assembled in a highly coordinated manner to ensure proper functioning of the complex (2). At the heart of the complex is a heterodimer of the D1 (the psbA gene product) and related D2 (the psbB gene product) polypeptides, which binds most of the redox-active cofactors involved in PSII electron transfer (3). The isolated PSII reaction center (RC) complex contains, in addition to D1 and D2, the intrinsic PsbI subunit and cytochrome b559, which bind to D1 and D2, respectively (4). Surrounding the PSII RC complex are two chlorophyll-binding proteins, CP43 (the psbC gene product) and CP47 (the psbB gene product). D1 and CP43 also provide several amino acid ligands to the Mn4Ca cluster involved in the oxidation of water (5, 6).

In vivo, the D1 protein exhibits much faster synthesis and degradation (or protein turnover) than the other PSII subunits (7). This feature reflects the operation of a PSII repair cycle to overcome the effects of PSII photoinhibition, which primarily causes irreversible damage to D1 (8). In most PSII-containing organisms, D1 is synthesized as a precursor protein (pD1) with a carboxyl-terminal extension (9). In the cyanobacterium Synechocystis sp. PCC 6803 this extension consists of 16 amino acid residues, which is removed in two steps by the CtpA endoprotease: first by cleavage on the carboxyl side of residue Ala1552 to generate i-D1, and then by cleavage after Ala1344 to form mature D1 (10–13).

PSII assembly appears to occur in a stepwise fashion and involves the formation of a PSII RC-like complex, possibly from D2-cytochrome b559 and pD1-PsbI pre-complexes (14, 15). Then the antennae, CP47 and CP43, plus other low-molecular mass polypeptides, become sequentially attached to form the PSII non-oxygen-evolving core complex. Light-driven assembly of the Mn4Ca cluster, and attachment of the luminal exposed extrinsic proteins of PSII, complete the assembly of the PSII holoenzyme, which exists as both a monomer and dimer in PSII reaction center containing CP47; HCF, high chlorophyll fluorescence; WT, wild type; Tes, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethylamino)ethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; PVDF, polyvinylidene difluoride.
vivo (2, 16, 17). Many details, such as the timing of co-factor attachment to apoproteins and binding of small membrane polypeptides to PSII, are still obscure, as are the identity and roles of PSII assembly factors.

Analysis of high chlorophyll fluorescence (HCF) photosynthetic mutants of Arabidopsis thaliana led to the identification of a nucleus-encoded assembly factor, HCF136 (now designated YCF48), which was targeted to the lumen and was needed for accumulation of PSII (18). Later, in vivo radiolabeling experiments demonstrated that formation of the PSII RC complex was specifically blocked in the absence of YCF48 (19). Furthermore, affinity chromatography using immobilized YCF48 suggested its interaction with a PSII assembly complex containing at least D2 and cytochrome b559 but whether it formed a complex with PSII in vivo was unclear (19).

YCF48 homologues are found in all oxygenic photosynthetic organisms including cyanobacteria. Somewhat surprisingly, the YCF48 homologue is not absolutely required for photoautotrophic growth of Synechococcus PCC 7002 and hence is not required for assembly of PSII (20). Whether YCF48 homologues play an evolutionarily conserved role in PSII assembly is therefore unclear, and an important point to resolve.

The homologue of YCF48 in the cyanobacterium Synechocystis PCC 6803 is encoded by the slr2034 gene (Cyanobase annotation). It is located upstream of the psbEFLJ operon, encoding four small subunits of PSII, including the two apoproteptides of cytochrome b559. In this article we investigated whether the slr2034 gene product (designated here as YCF48) is involved in the assembly of cyanobacterial PSII. We show that YCF48 is a component of PSII RC-like assembly complexes consisting of at least D2, D1, cytochrome b559, and PsbI (but lacking CP47 and CP43) and that it is required for efficient assembly of PSII. Importantly we provide evidence that the unassembled precursor proteins of D1 (pD1 and iD1) are binding partners for this assembly factor and that YCF48 also functions in PSII repair.

EXPERIMENTAL PROCEDURES

Construction and Cultivation of Cyanobacterial Strains—The strains used in the study were derived from the glucose-tolerant strain of Synechocystis sp. PCC 6803 (21) referred to as wild type (WT). The following previously described strains were used in the study: (i) the CP43-less strain, psbC−, with the psbC gene inactivated by a kanamycin-resistance cassette (22); (ii) the CP47-less strain, psbB−, with the psbB gene inactivated by a spectinomycin-resistance cassette (22); (iii) the psbEFLJ− strain with the psbEFLJ operon replaced by a kanamycin-resistance cassette (24).

The ycf48− strain was constructed by interrupting the slr2034 gene with a chloramphenicol-resistance cassette at the HindIII site located 506 bp into the 1026-bp open reading frame. Transformants were selected and segregated on chloramphenicol-containing agar plates containing 5 mM glucose and full segregation of the mutation was confirmed by PCR. Multiple ycf48− interruption strains were obtained by transformation of single mutants lacking psbC, psbB, and psbEFLJ genes using chromosomal DNA from ycf48− and their selection for the additional resistance to chloramphenicol.

Liquid cultures (100–200 ml) were grown in BG-11 medium in 500-ml Ehrenmeyer flasks, aerated using an orbital shaker, irradiated with 30 μmol photons m−2 s−1 of white light at 29 °C, and used when they reached a chlorophyll (Chl) concentration of about 2–3 μg ml−1. Solid medium contained in addition 10 mM Tes/NaOH, pH 8.2, 1.5% agar, and 0.3% sodium thiosulfate (24). Media for cultivation of non-autotrophic strains contained in addition 5 mM glucose.

Measurements of light-limited autotrophic growth rates were performed in microtitration plates (culture volume 0.25 ml) under intensive shaking and illumination at 25 μmol photons m−2 s−1. Optical densities at 750 nm were measured every 6 h using a microplate reader (Tecan Sunrise, Vienna, Austria). Values plotted against time were used for calculation of the doubling time.

RNA Isolation, Reverse Transcription, and Quantitative PCR—Total RNA was isolated from frozen cells following the hot TRIzol protocol (25), purified by RNeasy MinElute Cleanup Kit (Qiagen), and treated with TURBO DNase (Ambion). Twenty nanograms of purified RNA was used for cDNA synthesis using random primers and SuperScript II Reverse Transcriptase (Invitrogen). Real-time quantitative PCR were performed on the Rotor-Gene 3000 using the iQ SYBR Green Supermix (Bio-Rad). Each quantitative PCR experiment was performed on two replicates for two independent RNA isolations from the same culture. rps1 (encoding the 30S ribosomal protein, S1) was used as a reference gene. Its level was found to be proportional to total RNA (measured spectrophotometrically) in all strains. The ∆Ct method was used to calculate psbA gene expression normalized against rps1. The ∆Ct values were reproducible to within 0.5 cycle.

Luminometric and Polarographic Methods—The Fv/F0 parameter and kinetics of Chl variable fluorescence decay were measured in dark-adapted cultures (2.5 μg Chl ml−1) using a modulation PAM101 fluorometer (Walz, Effeltrich, Germany) with an ED-101US cuvette and the Dual Modulation Kinetic Fluorometer (Photon Systems Instruments, Brno, Czech Republic), respectively (26). The light-saturated steady-state rate of oxygen evolution in cell suspensions was measured polarographically in BG-11 medium containing 10 mM Hepes/NaOH, pH 7.0, using 0.5 mM p-benzoquinone and 1 mM potassium ferricyanide as artificial electron acceptors. Photosynthetic thermoluminescence was measured with cells filtered onto the nitrocellulose membrane (6 μg of Chl) using a commercial apparatus (Photon Systems Instruments, Brno, Czech Republic). Cells were covered with 25 mM Hepes/NaOH, pH 7.5, and dark-adapted for 5 min at 25 °C. Then, the temperature was decreased to 3 °C, two saturating flashes were given and thermoluminescence was measured during heating from 3 to 70 °C. The area of the thermoluminescence band B originating from the charge recombination between the donor S2 and S3 states and the PSII acceptor Qb (26) was calculated and taken as a measure for PSII content.

Preparation of Membranes and Their Protein Analysis—Cyanobacterial membranes were prepared by breaking the cells using glass beads (27) with the following modifications: cells were washed, broken, and finally resuspended in 25 mM Mes/NaOH, pH 6.5, containing 10 mM CaCl2, 10 mM MgCl2, and
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25% glycerol. Highly purified thylakoid and cytoplasmic membranes were obtained using the procedure described in Ref. 28.

For analysis of protein complexes, isolated membranes were solubilized with dodecylmaltoside (DM) (DM/Chl = 40, w/w) and analyzed by blue native electrophoresis at 4 °C in 5–14% polyacrylamide gels according to Ref. 29. Samples with the given Chl content (usually 6 μg for gel staining and 1 μg for Western blot) were loaded onto the gel. The protein compositions of the complexes were assessed in the second dimension by electrophoresis in a denaturing 12–20% linear gradient polyacrylamide gel containing 7 m urea (30). The whole lane from the native gel was excised, incubated for 30 min in 25 mM Tris/HCl, pH 7.5, containing 1% SDS (w/v) and placed on the top of the denaturing gel; two lanes were analyzed in a single denaturing gel. Proteins separated in the gel were either stained by Coomassie Blue or transferred onto PVDF membrane. Membranes were obtained using the procedure described in Ref. 28.

Pelleted cells or membranes were extracted with 100% methanol and Chl content in the extract was calculated from the absorbance at 666 and 720 nm (34).

RESULTS

YCF48 Is a Component of PSII RC Assembly Complexes in Synechocystis 6803—In A. thaliana, YCF48 was proposed to act as an assembly factor that is transiently bound to PSII assembly intermediate(s), but is not a component of fully assembled monomeric and dimeric core complexes (19). To test whether this conclusion also applies to the cyanobacterium Synechocystis sp. PCC 6803, we screened for the presence of the YCF48 homologue in various PSII complexes and assembly intermediates using a combination of two-dimensional PAGE (consisting of blue native PAGE and denaturing PAGE; two-dimensional BN/SDS-PAGE) and immunodetection using an antibody raised against the plant YCF48 (18). In WT, PSII proteins are found in monomeric (RCC(1)) and dimeric (RCC(2)) core complexes (Fig. 1). Small amounts of the PSII proteins, D2 and D1, also accumulate in two smaller PSII complexes: a core subcomplex lacking CP43 (termed RC47) and a RC-like assembly complex (RCa) lacking both CP47 and CP43 (15, 17). YCF48 could not be detected in RCC(1) and RCC(2) and was found to be mainly present in the low-molecular weight region, probably in an unassembled state. A second minor cross-reaction was, however, detected in the region of the RCa complex, suggesting that YCF48 might be a component of this complex (Fig. 1).

To test this hypothesis we screened for the presence of YCF48 in the PSII assembly intermediates that accumulate in a strain lacking CP47 (psbB−), which is only able to assemble PSII up to the stage of PSII RC-like complexes. Three different RC-like complexes with different electrophoretic mobilities, termed RC+, RCa, and RCb, have been identified in membranes of psbB−using BN/PAGE (Fig. 2, see also Ref. 15). Their precise subunit composition is unclear but each contains D2, either D1 or its processing intermediate iD1, both subunits of cytochrome b599, and PsbL, and each lacks both CP43 and CP47. In freshly prepared membranes of psbB−, the YCF48 protein was detected in the most abundant complexes, RC+ and RCa (Fig. 2). The remaining complex, RCb, was present in too low amounts for reliable detection of YCF48 (see also Ref. 15), but after sub-
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![Image](https://example.com/image.jpg)

**FIGURE 1.** Localization of YCF48 in photosystem II complexes of *Synechocystis* 6803 wild-type strain. Thylakoid proteins from wild-type strain (WT) grown in the presence of 5 mM glucose were separated by two-dimensional BN/PAGE, and either stained with Coomassie Blue (Coomassie stain) or blotted onto PVDF membrane and immunodecorated with antibodies raised against either D1, to detect D1 and i-D1, or YCF48. Designation of complexes: RCC(1), dimeric and monomeric PSII core complexes, respectively; PSI(3) and PSI(1), trimeric and monomeric PSII core complexes, respectively; RC47, PSII core complex lacking CP43; RCa, reaction center complex; U.P., low-molecular mass region of the blue native gel containing mainly unassembled PSII subunits. 2 μg of Chl was loaded for each sample.

jecting thylakoids to multiple freeze/thaw cycles, its content markedly increased. Subsequent immunoblotting failed to detect YCF48 in Rcb. Thus, we conclude that RC* and RCA differ from Rcb by the presence of YCF48 and that RC* most probably contains one or more, as yet unknown, protein components. The data also indicated that Rcb was probably generated from RC* and RCA by loss of additional factors including YCF48.

The *psbEFLJ* deletion mutant, designated *psbEFLJ*−, is unable to synthesize cytochrome b_{559} and as a consequence is unable to accumulate the D2 protein (17). This strain contains no PSII RC complexes and, in agreement with the conclusions above, only unassembled YCF48 could be detected in the *psbEFLJ*− strain (Fig. 2C).

**FIGURE 2.** Immunodetection of D1 and YCF48 proteins in the CP47-less mutant, *psbB*−, and the *psbEFLJ*− mutant. Thylakoid proteins from the *psbB*− mutant (panels A and B) and the *psbEFLJ*− mutant (panel C) were analyzed by two-dimensional BN/PAGE, blotted onto PVDF membrane, and immunodecorated sequentially with first antibodies raised against D1 and then against YCF48. Either freshly made thylakoids (panels A and C) or thylakoids subjected to several rounds of freeze-thaw (panel B) were analyzed. The analysis was performed on three different blots with different degrees of exposure; therefore the intensities of the signals cannot be directly compared. Designation of complexes: RC*, RCA and Rcb indicate three forms of the PSII RC-like complexes. 4 μg of Chl was loaded for each sample.

Absence of YCF48 Inhibits Formation and Accumulation of Cyanobacterial PSII RC Assembly Complexes—To test the physiological role of YCF48 in *Synechocystis* 6803, we constructed an insertion mutant (designated *ycf48*−) in which the *slr2034* gene was interrupted by a chloramphenicol-resistance cassette. Unlike the previously constructed inactivation mutant of *Synechococcus* PCC 7002 (20), the *Synechocystis* insertion mutant exhibited a significantly slower rate of autotrophic growth than WT under the growth conditions used (Table 1).

The yellow-green color of the culture suggested the enhanced accumulation of carotenoids in the mutant. This was supported by an increased absorbance at 440–500 nm in whole cells (supple-
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TABLE 1
Characteristics of WT and ycf48− grown under autotrophic conditions

|          | Doubling time | Oxygen evolution | PSII activity (Hill reaction) | Fv/Fm | TL band |
|----------|---------------|------------------|------------------------------|-------|---------|
|          | h             | µmol O₂ mg Chl⁻¹ h⁻¹ | µmol O₂ mg Chl⁻¹ h⁻¹ | relative area |
| WT       | 8.7 ± 0.1     | 202 ± 12         | 690 ± 122                   | 0.75 ± 0.02 | 32 ± 8  |
| ycf48−   | 16.4 ± 0.4    | 180 ± 12         | 450 ± 117                   | 0.53 ± 0.03 | 18 ± 2  |

* Measured in microtitration plates at an irradiance of 25 µmol photons m⁻² s⁻¹; average of 20 measurements ± S.D.

Light-saturated rate of oxygen evolution measured in the absence of artificial electron acceptors; average of 3 measurements ± S.D.

Light-saturated rate of oxygen evolution measured in the presence of 1 mM p-benzoquinone and 5 mM potassium ferricyanide; average of 10 measurements ± S.D.

Values obtained by PAM modulated fluorimeter in the presence of 10⁻⁵ M DCMU; average of 3 measurements ± S.D.

Values were obtained by integrating the area below the thermoluminescence glow curve (B band) measured in the range from 3 to 70 °C with the cells containing 6 µg of Chl; average of 3 measurements ± S.D.

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FIGURE 3. Two-dimensional BN/SDS-PAGE of radioactively labeled membrane proteins from WT and ycf48−. Cells of WT (left panels) and ycf48− (right panels), cultivated in the absence of glucose, were radiolabeled at 500 µmol µmol photons m⁻² s⁻¹ and 29 °C with a mixture of [35S]Met/Cys for 20 min and the labeled cells were used for isolation of thylakoids, which were analyzed by two-dimensional analysis of the D1-less strain of PsbA (supplemental Fig. S3).

 Minor differences in the accumulation of PSII complexes in WT and ycf48− were also detected by immunoblotting (Fig. 3, Bots). This technique was able to detect a small amount of the RCa complex in WT, but not in the mutant. The antibody against YCF48 confirmed the binding of this protein in the region containing RCa and also confirmed the absence of full-length protein in ycf48−. Finally, the apparent co-migration of unassembled α subunit of cytochrome b₅₅₉ and unassembled D2 subunit in the first dimension of the BN/PAGE gel analysis of ycf48− suggested the possible accumulation of a D2-cytochrome b₅₅₉ pre-complex, as identified previously in plant etioplasts (14). The existence of this pre-complex in Synechocystis was confirmed by co-immunoprecipitation of both subunits of cytochrome b₅₅₉ and D2 from a D1-less strain (psbA−), using antibody raised against the β subunit of cytochrome b₅₅₉ (Fig. 4). In addition, the existence of the complex is also indicated by the shape of the cytochrome b₅₅₉ signal in the previously published two-dimensional analysis of the D1-less strain of Synechocystis 6803 (17).

We also constructed a series of double mutants in which the ycf48 gene was inactivated in strains lacking the large PSII subunits: CP43, CP47, or D2. In the strain lacking CP43 (psbC−), PSII assembly could not progress beyond formation of the RC47 complex and the strongly radiolabeled D1 protein was amounts of the different types of PSII complex were also similar in ycf48−. In both strains, 80–90% of the D1 subunit was found in the dimeric and monomeric core complexes, RCC(2) and RCC(1), as assessed by the intensity of the Coomassie Blue-stained bands, with the remaining 10 to 20% in the RC47 complex (Fig. 3).

To characterize the PSII assembly process in WT and the ycf48− mutant, we pulse-labeled cells with [35S]Met/Cys and analyzed the incorporation of radiolabel into unassembled PSII subunits as well as those assembled in the different types of PSII complex (Fig. 3, autorad). In this respect, a striking contrast was observed between the WT and ycf48−. For WT the D1 protein was preferentially labeled in all PSII complexes including RCC(2), RCC(1), and RC47. No RC complexes or unassembled D1 forms were found in the strain, which is consistent with the very efficient incorporation of newly synthesized D1, and other PSII proteins, into PSII complexes under the conditions used for the labeling. In contrast, in the ycf48− mutant, more than 80% of the radiolabeled PSII proteins, CP47, CP43, D2, and D1 were present in the “unassembled” fraction of proteins (Fig. 3, autorad, ULP).
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FIGURE 4. Detection of the D2/cytochrome b559 pre-complex by co-immunoprecipitation. Pulse-labeled thylakoid proteins (5 μg of Chl) from the psbA− strain lacking D1 were co-immunoprecipitated using antisera specific for PsbF (the β-subunit of cytochrome b559) co-immunoprecipitation (co-IP anti-PsbF) or preimmune antiserum (co-IP pre) and the immunoprecipitate together with thylakoids from psbA− (TM) was analyzed by SDS-PAGE, blotted onto PVDF membrane, autoradiographed (left panel, Autorad), and then probed sequentially with antibodies against PsbE, PsbF, and D2 (Blot).

almost exclusively found in this complex (Fig. 5A). Additional interruption of ycf48 resulted in a remarkable suppression of D1 labeling in the RC47 complex, so that it only slightly exceeded the labeling of D2. This result, together with the increased amount of radiolabeled D2 in the unassembled protein fraction, again indicated limitation in the availability of the newly synthesized D1 in the psbC− ycf48− double mutant (Fig. 5B). Consequently, the accumulation of the RC47 complex in this strain was very limited as the complex was undetectable in the stained gel (supplemental Fig. S4B).

The CP47-less deletion strain, psbB−, has already been shown to accumulate the RC complexes RC*, RCa, and RCB with nearly all labeled D2 and about 30% of labeled D1 found in RCa (Fig. 5C and Ref. 17). In contrast, the formation of RC complexes in psbB− ycf48− was completely inhibited, as judged by radioactive pulse labeling (Fig. 5D) and immunoblotting (supplemental Fig. S4D). These data therefore indicated a crucial role for YCF48 in the accumulation of RC complexes in this strain.

Slr2034 Is Located in the Thylakoid Membrane—Based on the biochemical detection of D1, D2, and cytochrome b559 in cytoplasmic membrane fractions isolated by two-phase partitioning, a PSII RC complex consisting of these subunits has been proposed to be assembled in this membrane compartment (35), which then migrates to the thylakoid to complete its assembly. Because YCF48 is associated with various RC complexes, we tested for its presence in highly purified thylakoid and cytoplasmic membranes (36). Immunoblotting revealed the association of YCF48 with the thylakoid fraction but not the cytoplasmic membrane fraction (supplemental Fig. S5).

YCF48 Interacts with the Unprocessed Forms of D1—Radio-labeling of PSII proteins in WT suggested that YCF48 affected the maturation of pD1 (Fig. 3 and supplemental Fig. S3). Similarly, analysis of the double mutant, psbEFLJ− ycf48−, indicated the presence of only small amounts of unassembled mature D1, whereas incompletely processed D1 forms could be easily detected in the single mutant, psbEFLJ− (supplemental Fig. S6). Given that D2 does not accumulate in the psbEFLJ− strain (17), these data suggested that YCF48 influenced the maturation of pD1, before any interaction of pD1 with D2. Interestingly, accumulation of CP43 and CP47 was also suppressed in the psbEFLJ− ycf48− mutant (supplemental Fig. S6, A and B), although their synthesis did not seem to be affected by interruption of ycf48 (supplemental Fig. S6, C and D).

Overall the data thus far indicated a role for YCF48 in the accumulation of the precursor forms of D1 in vivo. To test whether YCF48 had the potential to interact directly with pD1, yeast two-hybrid analyses were performed using the so-called split ubiquitin system (32, 33). This system enables the interactions of heterologously expressed membrane proteins to be assessed in yeast cells by following reporter gene activation, leading to histidine prototrophy. As shown in Fig. 6, reporter gene product-dependent growth was observed after co-expression of YCF48 and the pD1 precursor protein as well as, although to a lesser extent, the iD1 version. Expression of mature D1, however, resulted in no growth of yeast cells, indi-
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![FIGURE 6. Yeast split ubiquitin assay of the YCF48-D1 interaction. Three independent transformants expressing YCF48, from plasmid pADSL2034, and the proteins indicated at the right margin, from plasmid pTM8V, were analyzed for HIS3 reporter gene activity by growth on medium either containing (+ His) or lacking histidine (−His).](image)

cating that, at least in yeast, YCF48 cannot recognize the fully C-terminal processed form of D1. This suggests that YCF48 binds to the C-terminal extension of the pD1 protein. In contrast, no interaction was detected with the D2 protein or unrelated proteins, such as the PsA subunit of photosystem I, or the yeast eR-located protein, Alg5 (33). Interestingly, the CtpA protease, which performs the C-terminal truncation of D1, was also not recognized by YCF48, suggesting that these two factors do not seem to physically interact during PSII biogenesis.

The D1 Extension Is Not the Sole Determinant for Binding of YCF48 to the PSII RC-like Complexes—The identification of an interaction of YCF48 with the precursor forms of D1, but not mature D1, raised the possibility that the C-terminal extension plays an important role in the binding of YCF48 during formation of the PSII RC-like complexes. To test this, we constructed a strain (D1-S345stop/psbB−) lacking both the D1 extension and CP47. Two-dimensional gel analysis of its membrane complexes showed that the RC* and RCA complexes still accumulated and both contained YCF48 (Fig. 7). Thus, it is probable that the YCF48 factor also interacts with the mature D1 but this interaction is too weak to be observed in yeast by the two-hybrid method.

The Absence of YCF48 Limits PSII Repair and the Fast Turnover of the D1 Protein—To find out if the absence of YCF48 affects the efficiency of PSII repair, we evaluated the time course of light-induced inhibition of oxygen evolution in WT and mutant cultures subjected to white light of 500 μmol photons m−2 s−1, either in the absence or presence of lincomycin. Although the rate of oxygen evolution in cells of WT declined only to about 60% of the initial level during the 3-h light treatment in the absence of lincomycin, this activity in cells of ycf48 mutant steadily decreased to 15–20%, with rather similar kinetics to that observed in the presence of lincomycin (Fig. 8A, left panel). Also recovery from photoinhibition under low light conditions, after a 1-h high light treatment, was very slow in ycf48− cells (Fig. 8A, right panel, closed symbols).

The inefficiency of PSII repair in ycf48− also correlated with the slow synthesis and degradation of the D1 protein, as assessed by pulse-chase labeling (Fig. 8B). This experiment revealed a half-life of D1 in WT of about 2 h (Fig. 8B, left panel), whereas in the mutant only about 40% of the protein was degraded during the 6-h chase (Fig. 8B, right panel). On the autoradiogram it was also apparent that D1 labeling was decreased in the mutant and that the more strongly radiolabeled D2 protein, which was found on the two-dimensional autoradiogram mostly in the region of unassembled proteins (Fig. 4, autorad), was more quickly degraded than in WT, probably because it could not be used for PSII assembly due to the low availability of the D1 protein caused by the absence of the YCF48 factor. Altogether, these data indicated that PSII repair and selective D1 turnover was also severely affected in the ycf48− mutant, most likely because of the reduced availability of newly synthesized D1 protein.

DISCUSSION

Role of YCF48 in the Formation of RC Complexes—Although YCF48 is known to be essential for formation of the PSII RC complex during assembly of PSII in Arabidopsis (19), the lack of an obvious PSII defect in a previously constructed ycf48 mutant of Synechococcus 7002 raised doubts about a role in PSII assem-

FIGURE 7. Immunodetection of D1, YCF48, and CP43 proteins in the psbB− mutant, psbB−, and in a double mutant additionally lacking the D1 extension (mutant D1-S345stop/psbB−). Membrane proteins from the CP47-less mutant, psbB−, and the double mutant lacking both the D1 extension and CP47 (D1-S345stop/psbB−) were separated by two-dimensional BN/PAGE, blotted onto PVDF membrane, and immunodecorated sequentially with antibodies raised against D1, CP43, and YCF48. The analysis of both mutants was performed on a single blot allowing direct comparison of protein signals; the CP43 signal (arrows) was used as an internal standard documenting equal loading of the sample. Designation of complexes are as described in the legend to Fig. 4.
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Figure 8. PSII repair under high and low irradiance (panel A) and degradation of the PSII proteins under high irradiance monitored by radioactive pulse-chase labeling (panel B) in the WT and ycf48 strains. Panel A, cells of WT (left panel) and ycf48 (right panel) were illuminated with 500 μmol photons m⁻² s⁻¹ of white light for 180 min in the absence (empty circles) or for 120 min in the presence (closed circles) of 100 μg ml⁻¹ lincomycin and PSII oxygen-evolving activity was assayed in whole cells. Values in the plots represent the mean of 3 measurements ± S.D. In the absence of lincomycin, a portion of the cells was transferred from high to low irradiance (50 μmol photons m⁻² s⁻¹) after 60 min and PSII oxygen-evolving activity was assayed for 2 additional h (empty squares). Initial values for WT and ycf48 were 600 ± 122 and 450 ± 117 μmol of O₂ mg of Chl⁻¹ h⁻¹, respectively. Panel B, cells of both strains were subjected to 250 μmol photons m⁻² s⁻¹ of white light for 20 min in the presence of [³⁵S]Met/Cys. Then the cells were washed, supplemented with unlabeled Met/Cys, and subjected to 500 μmol photons m⁻² s⁻¹ of white light for 6 h. Thylakoids were isolated, analyzed by SDS-PAGE, the gel stained (Gel stain), and the radioactive labeling of the proteins visualized using a PhosphorImager (Autorad). Quantification of radioactivity in the D1 band was performed by ImageQuant software with samples of each strain loaded on one gel. The radioactivity incorporated into the D1 band of each strain during the pulse was taken as 100%, numbers show means of three measurements, S.D. did not exceed 7%.

bly in cyanobacteria. Here we have confirmed that YCF48 is also not absolutely required for PSII assembly in the cyanobacterium Synechocystis 6803. However, we provide a variety of evidence to support an important physiological role for YCF48 in optimizing PSII assembly at the level of forming PSII RC assembly intermediates. Thus an important conclusion we draw is that YCF48 plays a role in PSII assembly in both cyanobacteria and chloroplasts.

In addition, we show for the first time that YCF48 associates with PSII RC complexes in vivo (Figs. 1 and 2). Komenda and colleagues (15) have recently reported the occurrence of three PSII RC complexes in a strain lacking CP47 and it was speculated that these complexes differ with respect to the presence of additional assembly factors not found in the final PSII complex. The present study provides compelling evidence that one of these additional assembly factors is YCF48. The absence of YCF48 in the RC47 complex suggests that YCF48 acts before binding of CP47 to the PSII RC complex. Our data also suggest that the smallest of the RC complexes, Rcb, does not contain YCF48, whereas the largest one, RC5, most probably contains additional unidentified protein components. Immunoblotting experiments failed to detect Psb27 in these complexes (data not shown), which is consistent with binding of this assembly factor later on in PSII assembly (37, 38). Thus, in summary, the heterogeneity of the RC complexes observed by two-dimensional BN/SDS-PAGE is partly related to binding of YCF48 to the PSII subunits D1 (id1), D2, cytochrome b₅₅₉, and PsbI.

Targets and Binding Partners of YCF48—Although PSII accumulates in the ycf48 mutant, radiolabeling experiments have revealed a drastic effect on the kinetics of assembly of PSII in the mutant. The radiolabeling pattern of PSII proteins in the ycf48 mutant is strongly reminiscent of the psbA mutant (compare Fig. 3 of this study and Fig. 5 in Ref. 17): both strains accumulate significant amounts of unassembled radiolabeled D2, CP43, and CP47. However, unlike psbA, the ycf48 strain contained reduced, but detectable, amounts of mature D1 in the unassembled protein fraction suggesting that D1 protein is synthesized but its utilization for the assembly of new RC complexes is very limited. Interestingly, reverse transcriptase-PCR analysis revealed an 8-fold increase in psbA mRNA in the ycf48 mutant compared with WT, possibly in response to the limitation in the availability of D1 protein.

A specific interaction of YCF48 with pD1 and (to a lesser extent) iD1 emerged from yeast two-hybrid analyses and is fully consistent with the analysis of the various ycf48 interruption strains. The absence of YCF48 not only resulted in rapid processing of pD1 but also led to a decrease in the overall level of labeled unassembled D1 protein in WT at low temperature (supplemental Fig. S3), in the psbB strain, lacking CP47 (Fig. 3C), and in the psbEFLJ strain lacking cytochrome b₅₅₉ and also unable to accumulate D2 (supplemental Fig. S6). Thus, these results suggest that YCF48 interacts with pD1 before its association with D2 to prevent its premature processing and degradation. In this respect, YCF48 is reminiscent of PsbI, which also stabilizes unassembled pD1 but remains associated with it throughout the assembly process (15). However, unlike PsbI, we were not able to identify the putative complex of pD1 and YCF48 by two-dimensional protein analysis, or by co-immunoprecipitation experiments, indicating a rather weak, and possibly transient, interaction between pD1 and YCF48.

Surprisingly, our characterization of the D1-extension-less strain lacking CP47 showed that the D1-extension is not the sole determinant for binding of YCF48 to the PSII RC-like complexes. It is possible that interaction of the newly synthesized precursor D1 with other assembly factors (for instance, PratA (39), or small PSII subunits absent in yeast cells (for instance, Psbl (15)), is needed for interaction of YCF48 with other parts of D1. It is also possible that the interaction between YCF48 and the D1 protein is sufficiently strong for detection by the two-hybrid methodology only when the extension (or part thereof) is present, whereas it is too weak and escapes detection in the case of the mature protein.

In A. thaliana YCF48 was proposed to interact with the D2-cytochrome b₅₅₉ pre-complex rather than with D1 (19). However, the similarity in the electrophoretic mobilities of labeled D2 and pD1 in Arabidopsis did not allow the unambiguous assignment of D2 or pD1 in the YCF48 affinity purified PSII subcomplex. Based on our present results we suggest that the complex previously isolated from Arabidopsis was not the
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D2-cytochrome $b_{559}$ pre-complex but the entire PSII RC complex containing pD1 instead of D1. Such an explanation would be in nice agreement with our analysis of the Synechocystis 6803 mutant.

In plant etioplasts, the presence of a D2-cytochrome $b_{559}$ assembly pre-complex has been previously reported (14). We have been able to show that a similar pre-complex is also able to form in Synechocystis, as documented by data obtained in the present study using strains lacking either the YCF48 homologue (Fig. 3) or the D1 protein (Fig. 4). Thus PSII RC assembly complexes seem to be able to form from smaller D1-PsbI-YCF48 and D2-cytochrome $b_{559}$ pre-complexes (Fig. 9), although we cannot at this stage rule out the operation of parallel assembly pathways.

Two-dimensional analysis of WT showed a fraction of YCF48 that is not assembled in any complex. Estimation of the YCF48 content in various mutants showed that, in the absence of fully assembled PSII, the accumulation of this protein factor is increased, with the highest level found in the strain lacking the FtsH (slr0228) protease (supplemental Fig. S7).

The reduced level of chlorophyll in the ycf48$^{-}$ strain (supplemental Fig. S1), and the impaired accumulation of CP43 and, especially, CP47 in the pseEFLJ$^{-}$/ycf48$^{-}$ strain (supplemental Fig. S6), indicate that the absence of YCF48 also affects other chlorophyll-binding proteins besides D1. Plücke$\text{en}$ et al. (19) detected an effect of the loss of YCF48 on the accumulation of PSII, but they concluded that the effect was indirect. As D1, PSI, CP43, and CP47 all bind chlorophyll, we cannot exclude the possibility that YCF48 is directly or indirectly involved in the distribution of chlorophyll among chlorophyll-binding proteins. Because the synthesis of D1 largely exceeds the synthesis of other chlorophyll-binding proteins, the effect of YCF48 on D1 synthesis, accumulation, and assembly would be much more detectable in comparison with other chlorophyll-binding proteins. Although yeast two-hybrid analyses failed to provide evidence for an interaction between YCF48 and other chlorophyll-binding proteins, the interpretation of this type of analysis should be done cautiously. Yeast cells do not contain chlorophyll and apoproteins lacking chlorophyll might be folded in a different way to proteins with bound pigment in the thylakoid.

**YCF48 Is Important for PSII Repair**—Besides a role in the assembly of PSII complexes, we have for the first time shown that YCF48 functions in the PSII repair cycle (Fig. 8) and hence is required for maintenance of functional PSII after it has been assembled. Given that YCF48 appears to be involved in the stabilization of pD1 in the membrane, the most likely reason for impaired D1 replacement in the ycf48$^{-}$ interruption strain is the reduced availability of pD1 needed for selective D1 replacement. The involvement of YCF48 in PSII repair provides an additional reason for the lack of PSII accumulation in the Arabidopsis hcf136/ycf48 mutant.

**Sites of PSII Assembly and Repair in Synechocystis 6803**—Early work by Zak et al. (35), based on the distribution of PSII subunits in membrane fractions isolated by two-phase partitioning, indicated the presence of PSII RC-like complexes in the cytoplasmic membrane fraction. This led to the proposal that the PSII RC was assembled in the cytoplasmic membrane then migrated into the thylakoid to complete its assembly into the PSII holoenzyme (35). In addition, the D1 processing protease, CtpA, was found exclusively in the cytoplasmic membrane fraction, so that D1 maturation had to occur here both during PSII assembly and PSII repair.

We have shown here that YCF48 is found predominantly in the thylakoid membrane fraction and, in addition, is part of two PSII RC assembly complexes (RC* and RCa). Our data on the location of YCF48 would therefore argue against a location for RC* and RCa in the cytoplasmic membrane. Possibly one role for YCF48 might be to participate in the movement of PSII pre-complexes and RC complexes from the cytoplasmic membrane to the thylakoid membrane.

However, there is still uncertainty about the role of the cytoplasmic membrane in PSII assembly and repair. The completely processed form of D1, iD1, is in our hands largely associated with PSII RC complexes (15, 17) but careful identification of unprocessed forms of D1 revealed their presence exclusively in thylakoids (40). Also, the selective degradation of D1 during PSII repair occurs most probably in the CP43-less complex, RC47. Both CP47, which is found in RC47, and the FtsH protease involved in D1 degradation, have been clearly localized to the thylakoid membrane fraction (35, 36). Cur-
rently the main evidence against PSII repair occurring in the thylakoid is the immunochemical detection of the CtpA prot
ease exclusively in the cytoplasmic membrane fraction (35). However, CtpA has not yet been detected in this compartment in proteomic analyses (28) and it is unclear whether the anti-
CtpA antibody used in earlier work is monospecific or whether it recognizes related proteins such as the CtpB and CtpC homologues.

Given the recent advances in understanding the structure of the thylakoid membrane in Synechocystis using electron-mic-
roscopic tomography (41), we suggest the possibility that assembly and repair of PSII may actually occur in laterally sep-
parated regions of the thylakoid, rather than in the cytoplasmic membrane, and that the cytoplasmic fraction isolated by two-
phased partitioning techniques might contain some “thylakoid”-derived membrane in addition to bona fide cytoplasmic membrane. Indeed recent fractionation experiments suggest that the cytoplasmic membrane fraction in Synechocystis is more heter-
ogeneous than originally thought (42). This proposed lateral heterogeneity in the thylakoid membrane system might, in some respects, be analogous to the lateral segregation seen in the thylakoid membrane system of chloroplasts.

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