Studies of *CrHCF244* reveal similarities and differences in *psbA* translation between *Chlamydomonas reinhardtii* and *Arabidopsis thaliana*

Xiaozhuo Wang[1], Guannan Wang[1,2], Lexi A. Cheramie[1], Cuiping Zhao[1], Maheshi Dassanayake[1], James V. Moroney[1] and David J. Vinyard[1,*]

[1] Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803

[2] Current address: Department of Biology, Stanford University, Palo Alto, CA, 94305

* Correspondence: dvinyard@lsu.edu, +1 225 578 0958

Highlight

We identified CrHCF244 as a translation factor of psbA in *Chlamydomonas*. Characterization of this protein and genetic examinations of other previously identified *psbA* translation factors in *Chlamydomonas* reveal similarities and differences in *psbA* translation between *Chlamydomonas* and *Arabidopsis*.

Abstract

Translation of *psbA*, the chloroplast gene that encodes the D1 subunit of Photosystem II (PSII), is important for both PSII biogenesis and repair. The translation of the *psbA* transcript in the chloroplast is under the control of nuclear gene products. Using a *Chlamydomonas* forward genetic screen and whole genome sequencing, we found a mutant defective in PSII activity and mapped the causative gene to be the homolog of *Arabidopsis High Fluorescence* (*HCF244*) gene, or *CrHCF244*. We then demonstrated that CrHCF244 is required for *psbA* translation in the alga, consistent with the function of HCF244 in *Arabidopsis*. The *Arabidopsis HCF244* gene also complemented the algal mutant. These results experimentally support the functional conservation of the homologs in green algae and land plants. However, these studies also revealed differences in *psbA* translation in *Chlamydomonas* and *Arabidopsis*. Loss of HCF244 in *Arabidopsis* results in a large decrease in chlorophyll. In contrast, there is no significant loss of chlorophyll in *Chlamydomonas* when *CrHCF244* is knocked out. This observation supports the uncoupling of D1 translation and chlorophyll association in algae as reported recently in an *ohp2* mutant, which is defective in chlorophyll delivery. Intriguingly, the *CrHCF244* mutant also exhibited a relatively high rate of suppressor mutants, pointing to the presence of alternative pathway(s) for D1 translation control. The characterization of both the conserved aspects and the differences in *psbA* translation control between algae and plants will help elucidate how this process is regulated.

Keywords

Photosystem II, *de novo* biogenesis of Photosystem II, Photosystem II repair, *psbA* translation, regulators of organelle genes, chlorophyll association

Abbreviations

PSII	Photosystem II
ROGE	regulators of organelle genes
HCF244	high chlorophyll fluorescence 244
At	*Arabidopsis thaliana*
Cr	*Chlamydomonas reinhardtii*
cTP	chloroplast transit peptide
cpPDC	chloroplast pyruvate dehydrogenase
DLA2	dihydrolipoamide acetyltransferase subunit 2
BN-PAGE	Blue native polyacrylamide gel electrophoresis
TF	translation factor

Introduction

Photosystem II (PSII) is a water-plastoquinone oxidoreductase that enables the capture and conversion of light energy into storable chemical energy. PSII is the first component in the photosynthetic electron transport chain that generates ATP and NADPH for CO_2 fixation. Though much is known about the subunit composition and structure of PSII (Shen, 2015), its biogenesis is less well understood. As a large protein complex containing over 20 subunits encoded by both nuclear and chloroplast genomes and containing various cofactors, the biogenesis of PSII is complicated (Nixon et al., 2010, Nickelsen & Rengstl, 2013, Lu, 2016). PSII biogenesis is a stepwise and highly coordinated process involving the synthesis of subunits, integration into the membrane, assembly of protein subunits, and association of cofactors (Nixon et al., 2010, Nickelsen & Rengstl, 2013, Lu, 2016). PSII is also subject to photoinhibition and a repair process is needed to restore its activity, during which the D1 subunit needs to be degraded and resynthesized (Järvi et al., 2015).

Translation of chloroplast genes encoding subunits of photosynthetic complexes including PSII have been found to be widely regulated by nuclear gene products, which are termed as regulators of organelle genes (ROGEs). As the gene expression in chloroplasts occurs prevalently at post-transcriptional levels, including mRNA stabilization and/or its translation, ROGEs are categorized as M and T factors, which refer to protein factors involved in maturation or stabilization of the target mRNAs and translation regulation of the mRNAs, respectively. Genetic and biochemical approaches have identified many ROGEs in regulating PSII (e.g. Tba1 (Somanchi et al., 2005), RBP63/DLA2 (Ossenbuhl et al., 2002, Bohne et al., 2013), RBP40/RB38 (Schwarz et al., 2007), MBB1 (Vaistij et al., 2000), MBC1 (Cavaiuolo et al., 2017), TBC2 (Auchincloss et al., 2002)), PSI (e.g. TAA1 (Lefebvre-Legendre et al., 2015)), Cyt b_6f (e.g. MCG1 (Wang et al., 2015)), and ATP synthase (e.g. TDA1 (Drapier et al., 1992, Eberhard et al., 2011), MDA1 (Viola et al., 2019)). Recently a systematic screening and characterization discovered several gene specific ROGEs and master regulators responsible for regulating the expression of multiple ROGEs to be involved in biogenesis of photosynthetic machinery (Kafri et al., 2023).

Among PSII genes, the translation of *psbA* encoding the D1 subunit is important for both PSII de novo biogenesis and repair. *psbA* gene expression control has been extensively studied in cyanobacteria, algae, and plants (Mulo et al., 2012). A few protein factors have been identified as involved in *psbA* translation in *Chlamydomonas*. Mayfield and coworkers biochemically identified *psbA* mRNA binding proteins RB47, RB60, RB38, and RB55, named based on their molecular size (Danon & Mayfield, 1991). The gene for RB47 was later cloned as a polyadenylate (poly (A)) binding protein, thus also named as chloroplast poly (A) binding protein cPAB1 (Yohn et al., 1998a, Yohn et al., 1998b). Though chloroplast mRNAs generally don't have poly (A) tails, there are A-rich regions in the UTRs, which could be potential binding sites. RB60 is a disulfide isomerase protein (Kim & Mayfield, 1997) which may regulate the redox state of RB47 and thus control its binding to *psbA* mRNA and the translation (Kim & Mayfield, 2002). Tba1, identified in a genetic screen, is a thioredoxin protein regulating *psbA* translation (Somanchi et al., 2005). Genetic screens by the Mayfield group have also found several *Chlamydomonas* nuclear mutants defective in *psbA* translation, some of which the causative genes are not mapped including F35 (Girard-Bascou et al., 1992, Yohn et al., 1996). Besides the dedicated protein factors involved in *psbA* translation, a "moonlighting" regulator was also identified. The dihydrolipoamide acetyltransferase subunit 2 (DLA2), a subunit of chloroplast pyruvate dehydrogenase complex (cpPDC) is involved in D1 translation regulation under mixotrophic growth condition (Bohne et al., 2013, Neusius et al., 2022, Ossenbuhl et al., 2002). DLA2 switches its role between acetate metabolism as a subunit of cpPDC, and regulator of D1 translation dissociating from the cpPDC complex and associating with thylakoid membrane through acetylation of its K197 residue (Neusius et al., 2022).

In land plants, two protein factors have been identified to be involved in *psbA* translation: High Chlorophyll Fluorescence proteins 173 and 244 (HCF173 and HCF244) (Schult et al., 2007, Link et al., 2012, Chotewutmontri & Barkan, 2020). Both are members of the short-chain dehydrogenase superfamily (Oppermann et al., 2001, Kavanagh et al., 2008, Moummou et al., 2012). HCF173 is required for *psbA* translation initiation and mRNA stability (Schult et al., 2007). HCF244, co-expressing with HCF173, is also involved in *psbA* translation, possibly working together with HCF173, as the double mutant has a more reduced plant size and fewer numbers of leaves (Link et al., 2012).

In this study, we used forward genetics to identify a PSII defective mutant in *Chlamydomonas* in which the causative gene was mapped to be a homolog of *Arabidopsis HCF244*. By examining the mutant, we provide evidence for its role in D1 translation in this alga. We show chlorophyll accumulation is not affected in the mutant, supporting the uncoupling of D1 translation and chlorophyll association in algae (Wang et al., 2023a). Intriguingly, we observed a number of suppressor mutants of the *CrHCF244* mutant that showed partial recoveries of PSII activity. In an effort to examine the relationship of CrHCF244 to the previously identified translation activators RB60 and RB47 in *Chlamydomonas*, we generated the corresponding mutants using CRISPR-Cas9 but no effect on PSII activity was observed in those mutants. This study supports the functional conservation of the plant ortholog HCF244 in alga. Suppressor mutants suggest an alternative pathway of D1 translation control. Genetic studies add to our understanding of the components and their roles in D1 translation control.

Materials and methods

Strains and culture conditions

Chlamydomonas strain CC-4533 (Zhang et al., 2014) and *psbA* deletion strain Fud7 (Bennoun et al., 1986) were obtained from the *Chlamydomonas* Resource Center (CRC). CC-4533 is the background strain for the CLiP library. We refer to CC-4533 as WT here. The *ami6* mutant and the CRISPR-Cas9 knockout mutants (Δ*CrHCF244-T23*, Δ*RB47*, Δ*RB60-1, 2, 3*) were generated in the background of CC-4533. Cells were grown in Tris-acetate-phosphate (TAP) (Harris et al., 2009) or high salt (HS) (Sueoka, 1960) media with shaking at 25ºC in an AlgaeTron incubator (Photon Systems Instruments). Growth tests were done by spotting or streaking cells onto TAP and/or HS plates with 1.5% (w/v) agar under white light at 60 µE m^{-2} s^{-1} intensity.

Chlamydomonas transformation

Electroporation was used in this study for the delivery of plasmid DNA (for generating random insertional mutants and complementation studies of mutants) and ribonucleoprotein (RNP) complexes (for generating CRISPR-Cas9 knockout mutants) into *Chlamydomonas* cells. Electroporation was done using MAX Efficiency™ transformation reagent (Invitrogen) according to the manufacturer's instructions. Briefly, cells from a 50 mL TAP culture with OD_{750} 0.3–0.5 were pelleted, washed twice with the transformation reagent and resuspended in 0.5–1 mL transformation reagent. For delivering plasmid, 110 µL of cell suspension was mixed with 1 µg plasmid DNA. For CRISPR Cas9 mutations, the 110 µL cell suspension was mixed with 10 µL Cas9-guide RNA (gRNA) RNP complex and 1 µg donor DNA. The mixture was added into a cuvette with 0.2 cm gap (Bio-Rad), and electroporated with Gene Pulser® II (Bio-Rad) at a voltage of 0.35 kV and capacitance of 25 µF. After electroporation, cells were kept at room temperature (RT) for 10 min, then transferred into 10 mL TAP with 40 mM sucrose and shaken in the dark overnight. Cells were then pelleted and spread onto selective plates. For complementation studies of *ami6* and for the Δ*CrHCF244-T23* mutant, plasmids C1 and C2 (see cloning construct) were electroporated into the mutants, and selected on HS plates.

Whole genome sequencing and analysis

Genomic DNA was extracted from CC-4533 and *ami6* mutant using the phenol-chloroform-isoamyl method. The isolated DNA was further treated with RNase to remove RNA contamination and quantified using a Qubit fluorometer. Library preparation and Illumina sequencing was performed by Genewiz (NJ, USA). After quality check with FastQC (v0.11.7, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), paired-end reads were aligned to *Chlamydomonas reinhardtii* CC-4532 reference genome (v 5.6 and v6.1) (Merchant et al., 2007) from Phytozome V13 using bowtie2 (v2.3.5) (Langmead & Salzberg, 2012) with the default settings. Aligned reads were sorted and deduplicated using Picard (v2.25.2, https://broadinstitute.github.io/picard). We subsequently employed GRIDSS2 structural variant caller (v2.13.2) (Cameron et al., 2021) to identify rearrangement break points that generally represent structural variant events using the recommended workflow. Results were organized into VCF (Variant Call Format) files, and then filtered using StructuralVariantAnnotation (v1.18.0, DOI: 10.18129/B9.bioc.StructuralVariantAnnotation) in R. The resulting structural variants were further validated and supplemented with the ones identified by GATK (v4.1) (O'Connor & van der Auwera, 2020) to generate the final list of variants. Lastly, the identified variants for the WT and *ami6* mutant were compared to identify these that were only present in the mutant.

Cloning constructs

Plasmid C1 was used for complementing *ami6* and CRISPR Cas9 knockout Δ*CrHCF244-T23* mutants with *CrHCF244* and the resulting transformants are *ami6-C1* and *T23-C1*. Plasmid C2 was used for complementing *ami6* and CRISPR Cas9 knockout Δ*CrHCF244-T23* mutants by *CrHCF244* with C-terminal tagged Venus and the resulting transformants are *ami6-C2* and *T23-C2.*

To construct the C1 plasmid, the open reading frame (ORF) of *CrHCF244* gene was amplified by Q5® High-Fidelity DNA Polymerase (New England Biolabs) using CC-4533 genomic DNA as template; the linear vector was PCR amplified using pLM006 (Mackinder et al., 2016) as template with primers in Table S1 and the mCherry and His tag sequences were excluded. To construct C2, the above ORF of *CrHCF244* was used and the linear vector was

PCR amplified using pLM005 (Mackinder et al., 2016) as template with primers in Table S1 (Venus sequence was included). The PCR products of the ORF of *CrHCF244* and linear vectors were assembled by Gibson assembly (Gibson et al., 2009) using Gibson Assembly® Master Mix (New England Biolabs). For the vector construct to transform the *Arabidopsis HCF244* gene into *Chlamydomonas*, the coding sequence for the HCF244 protein without the transit peptide was codon optimized (IDT Codon Optimization Tool) and then fused to the sequence of the *Chlamydomonas* PsaD transit peptide on the 5' end. The chimeric sequence was synthesized by Twist Bioscience. The synthesized fragment was used as template for PCR using primers in Table S1, after which it was assembled with the PCR product using primers in Table S1 from the backbone plasmid pLM006 (Mackinder et al., 2016), excluding mCherry and His tag sequence. Plasmids were verified by Sanger sequencing before transformation into *Chlamydomonas*.

Generating CRISPR-Cas9 knockout mutants in Chlamydomonas

CRISPR-Cas9 was used for targeted mutagenesis to knock out the *CrHCF244*, *RB47* and *RB60* genes in this study. The gRNA design, *in vitro* assembly with Cas9 to form RNP and delivery of RNP and donor DNA to cells were performed according to a published method (Picariello et al., 2020) with minor modifications. Briefly, the crRNAs targeting the second exon of *CrHCF244* (Cre02.g142146), the fourth exon of *RB47* (Cre01.g039300) and the first exon of *RB60* (Cre02.g088200) were designed on the website of Integrated DNA Technologies (IDT). The lack of off-targeting was verified by Cas-OFFinder (Bae et al., 2014). The crRNAs and tracrRNA (sequences were shown in Table S1), synthesized by IDT, were first annealed and then assembled with Cas9 (Alt-R™ S.p. Cas9 Nuclease V3, IDT) at 37°C for 15 min. Donor DNA for editing each gene was PCR amplified with primers in Table S1 using the pKS-aphVIII-lox aphVIII plasmid (Heitzer & Zschoernig, 2007) or the pHyg3 plasmid (Berthold et al., 2002) as templates to generate antibiotic-resistance cassettes flanked by 40-bp homology arms of the Cas9 cleavage site. The RNP and donor DNA was electroporated into CC-4533 after removal of cell wall by autolysin. Transformants were selected on TAP plates with 20 µg/mL paromomycin or hygromycin. Colonies were screened with PCR using primers in Table S1 and/or Sanger sequencing.

Spotting and streaking cells for growth tests

For spotting, TAP cultures with OD_{750} around 0.3–0.5 were used. Cells from 1 mL of culture were pelleted, washed with HS, and resuspended in 1 mL HS, and used for spotting. The OD_{750} was used to normalize the number of cells. 5 µL cultures with serial dilutions were spotted onto TAP and HS plates. For streaking, similar amounts of cells of each strain were inoculated onto the plates. Plates were kept under 60 µE m^{-2} s^{-1} at 25ºC for growth.

Chlorophyll fluorescence measurement

F_v/F_m was measured to indicate PSII activity (Genty et al., 1989). Cells in liquid cultures were measured with a double-modulation fluorometer (FL 6000, Photon Systems Instruments). Cells on plates were measured with a FluorCam (Photon Systems Instruments).

Confocal microscopy for subcellular localization of CrHCF244

Confocal microscopy of *Chlamydomonas* cells was done according to (Mackinder et al., 2016), using a Leica SP8 microscope. The Venus tagged strain (*T23-C2*) was grown in TAP to early exponential phase under light intensity of 60 µE m^{-2} s^{-1}. Around 8 µL of culture was mixed with an equal volume of 0.1% (w/v) low melting agarose on the slide and covered with a #1.5 cover slip. The observation was done using a 63x water immersion objective. White light laser at 512 nm was used for excitation of Venus fluorescence and emission between 520-540 nm was collected. Chlorophyll autofluorescence was excited at 561 nm and emission between 560-590 nm was collected as chlorophyll fluorescence.

Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and immunoblotting analysis

To analyze the abundance of photosynthetic proteins, *Chlamydomonas* cells were grown in liquid TAP under light intensity of 20 µE m^{-2} s^{-1}. The low light was used here to avoid possible secondary effects on the photosynthetic machinery in the mutants from high light intensity. Total protein was extracted using 2% SDS in 50 mM Tris-HCl (pH 8.0). A BCA assay was used to measure total protein concentration. Laemmli sample buffer was added to protein samples followed by incubation at room temperature for 10 min. Around 10 µg of protein was

loaded to home-made 12% Tris-Glycine polyacrylamide gels containing 6 M urea. After electrophoresis, proteins were transferred to a PVDF membrane and blocked with 5% non-fat milk. Antibodies of PSII subunits D1 (Agrisera, AS05 084), D2 (Agrisera, AS06 146), CP43 (Agrisera, AS11 1787), CP47 (PhytoAB, PHY0319) and PsbO (Agrisera, AS06 142), and antibodies against PSI subunit PsaA (Agrisera, AS06 172) and Cyt b_6f subunit Cyt f (a gift from Prof. Terry Bricker, LSU) were used. A rabbit polyclonal antibody against RB60 was made in this study using the RB60 peptide SEKMPPTIEFNQKNSDKIFN as the antigen by YenZym Antibodies. The PVDF membrane was incubated with diluted primary antibodies for 1 h at room temperature followed by washing and incubation with 1:20,000 diluted HRP-conjugated secondary antibody (Invitrogen, 31460) at room temperature for 1 h. ECL reagent (Bio-Rad, Clarity Western ECL Substrate) was applied to the membrane for chemiluminescence detection using a ChemiDoc XRS+ imaging system.

Crude thylakoid membrane isolation and Blue-native (BN) PAGE

Crude thylakoid membranes from *Chlamydomonas* were prepared as described (Garcia-Cerdan et al., 2019) with minor modifications. Cells were resuspended in lysis buffer (25 mM HEPES-NaOH, pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$ and 5% [w/v] glycerol) and passed through a French press at gauge pressure 700 psi, 4°C to lyse cells. The lysate was centrifuged at 3000×*g*, 5 min, 4°C to remove unbroken cells and cell debris. The supernatant was then centrifuged at 30,000 × *g*, 30 min, 4°C. The pellets are used as crude thylakoid membranes. After resuspension in solubilization buffer (25 mM HEPES-NaOH, pH 7.5, 50 mM NaCl and 5% glycerol) at Chl concentration of 0.5 μg/μL, the thylakoid membranes were solubilized with 1% α-DDM (GLYCON Biochemicals GmbH) on ice for 20 min (Tokutsu et al., 2012), followed by centrifugation at 20,000 × *g* for 10 min at 4°C to remove unsolubilized thylakoids. The supernatant was run on a 5-12% BN-PAGE gel as previously described (Wang et al., 2016).

Chlorophyll measurement

Chlorophyll measurement was done using methanol extraction, as previously described (Porra et al., 1989). Cells were grown in TAP under low light conditions, to avoid any possible secondary effects on the photosynthetic machinery in the mutants.

^{14}C-acetate pulse labeling

Pulse labeling of *Chlamydomonas* cells was done according to the literature (Calderon et al., 2023, Chua & Gillham, 1977, Spaniol et al., 2022). Briefly, 5 mL of TAP-grown cells under 20 µE m^{-2} s^{-1} were pelleted and washed with HS. After resuspension in 0.5 mL HS, cells were incubated under 20 µE m^{-2} s^{-1} with vigorous shaking for 1.5 h to deplete the cellular carbon pool. Then, 10 µg/mL cycloheximide was added to the culture to inhibit cytosolic translation followed by the addition of 20 µCi/mL ^{14}C-acetic acid, sodium salt (Moravek, MC200) and incubated for 20 min under 20 µE m^{-2} s^{-1}. The culture was diluted into 10 volumes of cold TAP with 50 mM nonradioactive acetate and cells were pelleted and total protein was extracted. Protein samples were run on 12-18% Tris-Glycine SDS-PAGE gels with 6 M urea and then transferred from the gel to a PVDF membrane (Amersham™). The membrane was then exposed to a storage phosphor screen (Molecular Dynamics) for three weeks and imaged with a Typhoon™ 8600 imager.

Results

Screen and mapping of a mutant ami6 defective in PSII activity

To look for protein factors involved in PSII biogenesis, random insertional mutagenesis was used to generate a pool of mutants. One mutant, *ami6*, was identified which could not grow photoautotrophically and has no PSII activity (Fig. 1A). To identify the candidate causative gene(s) in this mutant, we conducted whole genome sequencing. Analysis of the whole genome sequencing data using *Chlamydomonas reinhardtii* v6.1 (https://phytozome-next.jgi.doe.gov/info/CreinhardtiiCC_4532_v6_1) genome as the reference genome revealed a large deletion (~347 kb) was mapped onto chromosome 15 (Fig. 1B). This genomic variance was verified by PCR and Sanger sequencing (primer sequences are in Supplementary Table S1 and results are in Supplementary Fig. S1). There are 28 genes (Supplementary Dataset S1) located in the deleted genome region. One of the deleted genes, Cre02.g142146, a homolog of *Arabidopsis HCF244*, was able to restore photoautotrophic growth and PSII activity in *ami6* (Fig. 1A). Adding the fluorescent protein Venus tag at the C-terminal of CrHCF244 did not affect the function of the protein (Fig. 1A and 1C). Of note, after we confirmed the causative gene in *ami6* in late May of 2022, we noticed an annotation on this gene in *Chlamydomonas* genome v6.1, the newly released version of the genome, that F35, a previously identified nuclear mutant defective in PSII (Girard-Bascou et al., 1992, Yohn et al., 1996) is mutated in this gene. However, there's no publication regarding on the mapping of the gene in F35. Our identification, mapping and complementation of *ami6* mutant verified the genetic relation of *CrHCF244* with PSII in *Chlamydomonas*.

CRISPR-Cas9 to knock out CrHCF244

We next used CRISPR-Cas9 to generate a *CrHCF244* knockout mutant. The gRNA was designed to target the second exon of the gene, as illustrated in Fig. 2A. Based on PCR screening and Sanger sequencing (Supplementary Fig. S2A and Fig. S2B), transformant number 23 was selected, which is termed as Δ*CrHCF244-T23*. Δ*CrHCF244-T23* has a genomic variation at the Cas9 cleavage site in exon 2 of *CrHCF244*. For sequence alignment see Supplementary Fig. S2B. This mutation would result in the point mutation of F37 to G37 and a frame shift after the 37th residue followed by a premature early stop codon, which abolished the function of the gene.

Δ*CrHCF244-T23* could not grow photoautotrophically and has no PSII activity (Fig. 2B and C), consistent with the phenotype of *ami6*. Similar to *ami6*, *CrHCF244* restored the photosynthetic growth and PSII activity of Δ*CrHCF244-T23* (Fig. 2A). The complementation of both the *ami6* mutant and the CRISPR-Cas9 knockout mutant by *CrHCF244* verified that this gene is required for PSII biogenesis. The function is consistent with its homolog in plants (Link et al., 2012, Chotewutmontri et al., 2020).

PSII, specifically, is affected in ami6

To test the accumulation of photosynthetic complexes in the absence of CrHCF244, immunoblotting of subunits of PSII, PSI and Cytb6f was performed. As shown in Fig. 3A, PSII core subunits including D1, D2, CP43, CP47 were drastically reduced in the *ami6* mutant and Δ*CrHCF244-T23* mutant, whereas abundance of subunits of PSI and Cyt b_6f did not change in the mutants. The extrinsic subunit PsbO also showed a decrease in abundance, though to a lesser level than those core subunits. Introducing *CrHCF244* into the two mutants restored the accumulation of PSII subunits, as shown in the complemented lines (Fig. 3A). With respect of the PSII complexes, immunoblotting of the protein complexes separated on BN-PAGE showed that none of the PSII complexes accumulated to a significant level in *ami6* compared to WT (Fig. 3B and C). The results showed absence of CrHCF244 impaired both accumulation of individual subunits and assembly of PSII complexes.

psbA translation is affected in CrHCF244 mutants

We used ^{14}C-acetate pulse labeling to look at the synthesis of PSII subunits in *ami6* and the CRISPR-Cas9 mutants. Fig. 4A showed that in the mutants the synthesis of D1 subunit is not observable, whereas the synthesis of D2 and CP43 occurred similarly to WT. The synthesis of CP47 was absent in the mutants. This is due to the assembly-controlled translation that the presence of D1 is required for translation of CP47 (Minai et al., 2006). The transcription of *psbA* gene is not affected in the mutant (Fig. 4B). This indicates that the decrease of D1 synthesis is not caused by defects in transcription.

Arabidopsis HCF244 complements the ami6 mutant

To test whether the *Arabidopsis* homolog gene could complement the *Chlamydomonas* mutant, we made a construct in which the sequence of the transit peptide of PsaD from *Chlamydomonas* (CrPsaD) was fused with the sequence of the mature *Arabidopsis* HCF244 protein (Fig. 5A). The construct was then transformed into *ami6* and transformants were selected under photosynthetic conditions. The results (Fig. 5B) showed that *AtHCF244* can partially complement the *Chlamydomonas* mutant.

CrHCF244 localizes to chloroplast

Confocal microscopy was used to examine the subcellular localization of CrHCF244. The complementation strain *ami6-C2* (the same line used in Fig. 1A), which is the *ami6* mutant transformed by C2 construct (depicted in Fig. 1C) was used for microscopy. In this strain, fluorescent protein Venus was tagged at the C-terminus of CrHCF244. As shown in Fig. 6, the Venus signal mostly overlapped with chlorophyll signal, indicating that CrHCF244 is localized in the chloroplast. The photosynthetically restored strain by Venus-tagged CrHCF244 represents the functional and native localization of the protein. Venus tagged CrHCF244 in the background of WT showed the similar result (Wang et al., 2023b).

Chlorophyll did not decrease in the absence of CrHCF244

In plants, it is reported that apo-D1 translation and chlorophyll association are coupled and this coupling is mediated by a complex consisting of D1 translation factor HCF244 and chlorophyll delivery proteins, which are one-helix protein 1 and 2 (OHP1 and OHP2) (Hey & Grimm, 2018, Li et al., 2019b, Chotewutmontri et al., 2020). The protein accumulation of the components in the complex heavily depends on the presence of each other (Hey & Grimm, 2018). To test the effect of CrHCF244 absence on the accumulation of OHP2 in *Chlamydomonas*, we looked at chlorophyll content in the Δ*CrHCF244-T23* mutant. The total Chl and Chl a/b ratio are shown in Table 1. The minor change in chlorophyll amount suggest that OHP2 is still present at a significant level in Δ*CrHCF244-T23*, as the Chl amount decreased around 40% in the absence of OHP2 (Wang et al., 2023a).

Suppressor mutations suggests an alternative pathway of D1 translational control

Interestingly, we observed colonies originating from Δ*CrHCF244-T23* on HS plates. Growth and F_v/F_m measurements (Fig. 7A) showed these strains have partially restored PSII activity. PCR and Sanger sequencing of *CrHCF244* proved that the *CrHCF244* gene did not undergo secondary mutations in these colonies (Supplementary Fig. S3A and Fig. S3B), indicating the colonies are likely suppressor mutants that have mutations in other gene(s). This suggests an alternative pathway for D1 translation control. We were also able to observe suppressor mutants in *ami6*, in which 27 genes besides *CrHCF244* have been deleted. We propose the pathway for *psbA* translation control in Fig. 7B, in which the alternative pathway is mediated by an alternative translation factor (TF) that was repressed.

PSII is not affected in CRISPR-Cas9 mutants of RB47 and RB60

To examine the relationship of CrHCF244 with previously reported D1 translation factors RB47 and RB60, we used CRISPR-Cas9 to generate knockout mutants. The gene maps and Cas9-gRNA RNP cleavage sites are shown in Fig. 8A. We were able to get one mutant for RB47 and multiple mutants for RB60 from PCR screening of the transformants. Here, we used the one mutant of RB47, named as Δ*RB47* and three mutants of RB60, named as Δ*RB60-1*, Δ*RB60-2*, Δ*RB60-3*. PCR identification of the mutants are shown in Fig. S4A and Δ*RB47* was further verified by Sanger sequencing (Fig. S4B). Unexpectedly, we did not detect significant PSII activity or D1 accumulation defects in the mutants (Fig. 8B). Immunoblots against RB60 verified the knockout of *RB60* in its mutants (Fig. 8C). We did not succeed in raising an RB47 antibody. Our genetic analysis does not support the previously proposed roles of RB47 and RB60 in D1 translation (Yohn et al., 1998b, Kim & Mayfield, 1997, Kim & Mayfield, 2002). This discrepancy may be due to the fact that previous identifications were mostly based on biochemical data when targeted gene editings were not possible.

Discussion

Using forward genetics, we identified a mutant, *ami6*, that is defective in PSII (Fig. 1A). The high tendency of *Chlamydomonas* to randomly insert DNA fragments into its genome makes it very accessible for generating a mutant pool or library. Such genetic screenings have

facilitated the discovery of many protein factors involved in PSII biogenesis and repair including Tba1 (Somanchi et al., 2005), REP27 (Park et al., 2007), Alb3 (Bellafiore et al., 2002, Ossenbuhl et al., 2004, Gohre et al., 2006), and RBD1 (Calderon et al., 2013). Recently, high throughput genetic screening and mapping has resulted in many other potential photosynthesis genes to be identified (Wakao et al., 2021, Kafri et al., 2023, Li et al., 2019a, Fauser et al., 2022). To understand PSII biogenesis and identify factors involved in this process, we started by generating a mutant pool and screened it for photosynthetic growth defective mutants using chlorophyll fluorescence to test the PSII activity and found one mutant that doesn't have PSII activity (Fig. 1A). We then used whole genome sequencing of the mutant and its corresponding WT to map the genomic variation. Analysis of the sequencing data revealed an approximately 345 kb deletion on chromosome 2 (Supplementary Dataset S1) when using the *Chlamydomonas reinhardtii* v5.6 genome (https://phytozome-next.jgi.doe.gov/info/Creinhardtii_v5_6) as the reference genome in early 2022. Recently, we re-analyzed the whole genome sequencing data using *Chlamydomonas reinhardtii* v6.1 (https://phytozome-next.jgi.doe.gov/info/CreinhardtiiCC_4532_v6_1) genome as the reference genome and the deletion (~347 kb) was mapped onto chromosome 15 (Fig. 1B), due to the significant improvements in assembly quality of the v6.1 genome. Analysis of the whole genome sequencing showed a ~347 kb deletion, where there are 28 genes, on chromosome 15 in the *ami6* mutant (Fig. 1B). We pinpointed the causative gene to be *Cre02.g142146*, or *CrHCF244*, the homolog gene of *Arabidopsis HCF244* by complementation (Fig. 1A) and by reproducing the mutant phenotype using a CRISPR knockout (Δ*CrHCF244-T23*) of the same gene (Fig. 2).

Both *ami6* and Δ*CrHCF244-T23* have no PSII activity (Fig. 1A and Fig. 2A). Immunoblotting showed that major subunits of PSII are drastically reduced in both mutants, whereas other photosynthetic complexes are not affected (Fig. 3A). The specific effect of CrHCF244 on PSII is consistent with its homologs in plants (Link et al., 2012, Chotewutmontri et al., 2020). PSII biogenesis is a stepwise and highly coordinated process, and the accumulation of subunits is tightly regulated (Baena-Gonzalez & Aro, 2002, Nixon et al., 2010, Nickelsen & Rengstl, 2013). It has been established that the translation of PSII subunits is controlled by an assembly-controlled mode, termed as CES, in which presence of the D2 subunit is prerequisite for translation of D1, and presence of D1 is prerequisite for translation of CP47 (Wollman et al., 1999). The mechanism of CES is that the unassembled subunit will bind to the 5' UTR of its

own mRNA and the recruiting of the subunit, e.g. D1, by its assembly partner D2-Cyt b_{559} releases the translation repression (Minai et al., 2006). To know whether any defects in synthesis caused the reduced accumulation of PSII subunits, we used ^{14}C-acetate pulse labeling and observed that the synthesis of D1 is affected in the mutants (Fig. 4A). As expected, in the absence of D1, CP47 translation was abolished as well in the mutants (Fig. 4A). The data presented showed that CrHCF244 affects PSII biogenesis by affecting *psbA* translation, consistent with its role in land plants (Link et al., 2012, Chotewutmontri et al., 2020).

Due to the large differences in the *psbA* 5' UTR sequences (Shen et al., 2001) and no homolog of trans-regulating factors has been reported to be involved in *psbA* translation between *Chlamydomonas* and *Arabidopsis*, it was not clearly known whether *psbA* translation control is conserved in algae and plants. In this study, we show that *CrHCF244*, a homolog of *Arabidopsis HCF244* function in *psbA* translation, consistent with the function of *HCF244* in *Arabidopsis* (Link et al., 2012). The *Arabidopsis* ortholog complementation of the *Chlamydomonas* mutant (Fig. 5B) also demonstrated the functional conservation of this gene from algae to plants, consistent with the relatively high sequence identity (Fig. S5) of the proteins between the two organisms. The other *psbA* translation factor identified in *Arabidopsis*, HCF173 (Schult et al., 2007), is also possibly involved in *psbA* translation in *Chlamydomonas*. In a proteomics study, it was noted that D1 accumulation was reduced in a *CrHCF173* mutant (Kafri et al., 2023) which suggests it could be a translation factor for *psbA*. Previously RB47 and RB60, were biochemically identified as possible *psbA* translation factors in *Chlamydomonas*. But no *Arabidopsis* homologs of these proteins functioning in *psbA* translation have been identified. Genetic evidence in this study showed that RB47 and RB60 play no major role in *psbA* translation (Fig. 8B, 8C, 8D). These data also support the hypothesis that similar factors are being employed in *psbA* translation in the two organisms.

In *Arabidopsis*, *psbA* translation is coordinated with chlorophyll association to apo-D1 and the early assembly of D1, as is the case with cyanobacteria (Chidgey et al., 2014, Knoppova et al., 2014). The coupling of D1 translation and chlorophyll association is mediated by the HCF244-OHP1-OHP2 complex (Chotewutmontri et al., 2020, Li et al., 2019b, Hey & Grimm, 2018, Myouga et al., 2018) in *Arabidopsis*. When the *psbA* translation factor HCF244 is absent, the accumulation of OHP1 and OHP2, which bind and deliver chlorophyll to apo-D1, is

abolished as well. This defect in chlorophyll association affects the accumulation of chlorophyll possibly through chlorophyll biosynthesis. In *Arabidopsis*, when OPH1 or OHP2 is absent, *psbA* translation is abolished due to the loss of HCF244. However, recently, it was shown by ribosome profiling that D1 translation still occurred in the *Chlamydomonas ohp2* mutant, in which the absence of OHP2 affects the delivery of chlorophyll to apo-D1. The loss of OHP2 also affected the biosynthesis of chlorophyll, and chlorophyll decreased to 40% of WT levels. This result suggested D1 translation is uncoupled from chlorophyll association in *Chlamydomonas* (Wang et al., 2023a). The lack of chlorophyll changes in Δ*CrHCF244-T23* (Table 1) indicates OHP2 is still present at significant amounts. This contrasts with the presence of the HCF244-OHP1-OHP2 complex as proposed in plants. The lack of a chlorophyll change in a mutant defective in production of D1 also supports the uncoupling of the translation of apo-D1 and chlorophyll delivery in *Chlamydomonas* (Wang et al., 2023a).

Previously, it was proposed that RB60 regulates the redox state of RB47, promoting its binding to *psbA* mRNA and translation (Kim & Mayfield, 1997). Here we showed that neither of these proteins is essential for *psbA* translation using genetic evidence (Fig. 8). The previous conclusions were mostly based on *in vitro* biochemical data (Kim & Mayfield, 1997, Yohn et al., 1998a, Danon & Mayfield, 1991). There was no genetic study on RB60, and the genetic analysis on RB47 used mutants with reduced accumulation of RB47 but no mutation in the *RB47* gene (Yohn et al., 1998b, Bruick & Mayfield, 1999), due to the limitation in targeted mutagenesis of nuclear genes in *Chlamydomonas*. A previous scenario of unspecific binding of *psbA* mRNA was observed in which RB38, originally identified as a *psbA* mRNA associating protein (Danon & Mayfield, 1991), was later was shown to function in *psbD* translation control (Schwarz et al., 2007).

In this study, we confirmed that CrHCF244 is a *psbA* translation factor. A previous study identified Tba1 as a *psbA* translation factor (Somanchi et al., 2005). There was a suggestion that CrHCF173 is also a *psbA* translation factor based on the function of its *Arabidopsis* homology and proteomics detection of reduced D1 accumulation in the mutant (Kafri et al., 2023). Therefore, there are at least two or three proteins required for translation of *psbA in Chlamydomonas*, considering DLA2 is a moonlighting regulator, which only functions in the presence of acetate (Neusius et al., 2022, Bohne et al., 2013). Questions arise as to why multiple

components are required for the translation of one message and how the absence of each of them would abolish the translation. They could function in a complex with each of them required for the stability of the complex, like the translation initiation complex in the cytosol of eukaryotes (Jackson et al., 2010, Brito Querido et al., 2024). Alternatively, the translation initiation of *psbA* could be a sequential process and each of them is needed in a stepwise way. Regarding the working mechanism of CrHCF244 in *psbA* translation, this protein could directly bind to the 5' UTR of *psbA* or bind indirectly as mediated by other proteins. There are proteins in the SDR superfamily shown to be RNA binding proteins (Bollenbach & Stern, 2003). We tried to recombinantly express CrHCF244 and test its RNA binding ability *in vitro* but failed to get soluble expression of the protein in *E. coli*.

Our observation of suppressor mutants of Δ*CrHCF244* suggests the presence of an alternative complex for D1 translation or perhaps a step in the translation process could be bypassed in these lines. The inability of the Δ*CrHCF244-T23* mutant to grow photoautotrophically and the absence of PSII activity indicate this alternative pathway is in repression. We propose a possible scenario as illustrated in Fig. 7B. A suppressor mutation of a translation factor of *psbD* was reported and the mutation is in the 5' UTR of the *psbD* mRNA (Rochaix et al., 1989). We sequenced the *psbA* 5' UTR of these suppressors and there is no mutation, suggesting the mutation might involve nuclear gene product(s).

Supplementary data

Supplementary data for this manuscript is listed as below and available online.

Figure S1 The large deletion in *ami6* was verified by PCR and Sanger sequencing.

Figure S2 Genomic identification of Δ*CrHCF244-T23* mutant.

Figure S3 PCR and Sanger sequencing verification *CrHCF244* in the suppressor mutants of Δ*CrHCF244-T23*.

Figure S4 Genomic identification of CRISPR-Ca9 mutants of *RB47* and *RB60*.

Figure S5 Clustal Omega multiple sequence alignment of HCF244 from *Chlamydomonas* (CrHCF244) and *Arabidopsis* (AtHCF244).

Table S1 Primers and crRNA sequences used in this study.

Dataset S1 *Genomic variance and deleted gene list in ami6*

Acknowledgments

We thank Shared Instrumentation Facility at Louisiana State University for confocal microscopy support. We thank Prof. Terry Bricker for the Cyt f antibody. We thank Dr. Yuqing Hou at University of Massachusetts Medical School for her advice on CRISPR-Cas9 gene editing in *Chlamydomonas*. Biorender was used for making diagrams (Fig. 1C, Fig.2A, Fig. 5A, Fig. 7B and Fig. 8A)

Author contributions

Conceptualization: XW, JVM, DJV; Experiments: XW, LAC, CZ, JVM; Data analysis and interpretation: XW, GW, CZ, MD, JVM, DJV; Writing: original draft- XW, JVM, DJV, reviewing and editing- all authors; Supervision of study: DJV; Funding acquisition: DJV and MD.

Conflict of interest

The authors declare that they have no conflicts of interest.

Funding

This work is supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Science, Division of Chemical Sciences, Geosciences, and Biosciences, Photosynthetic Systems through Grant DE-SC0020119. This work was also supported by the US National Science Foundation IOS-EDGE 1923589 and US Department of Energy BER DE-SC0022985 grants for genomic data analysis.

Data availability

All relevant *Chlamydomonas* strains will be archived in Chlamydomonas Resource Center. All relevant data and other materials are available from the authors upon request.

References

Auchincloss AH, Zerges W, Perron K, Girard-Bascou J, Rochaix JD, 2002. Characterization of Tbc2, a nucleus-encoded factor specifically required for translation of the chloroplast psbC mRNA in Chlamydomonas reinhardtii. *J Cell Biol* **157**, 953-62.

Bae S, Park J, Kim JS, 2014. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics* **30**, 1473-5.

Baena-Gonzalez E, Aro EM, 2002. Biogenesis, assembly and turnover of photosystem II units. *Philos Trans R Soc Lond B Biol Sci* **357**, 1451-9; discussion 9-60.

Bellafiore S, Ferris P, Naver H, Gohre V, Rochaix JD, 2002. Loss of Albino3 leads to the specific depletion of the light-harvesting system. *Plant Cell* **14**, 2303-14.

Bennoun P, Spierer-Herz M, Erickson J, *et al.*, 1986. Characterization of photosystem II mutants of Chlamydomonas reinhardii lacking the psbA gene. *Plant Mol Biol* **6**, 151-60.

Berthold P, Schmitt R, Mages W, 2002. An Engineered Streptomyces hygroscopicus aph 7″ Gene Mediates Dominant Resistance against Hygromycin B in Chlamydomonas reinhardtii. *Protist* **153**, 401-12.

Bohne AV, Schwarz C, Schottkowski M, *et al.*, 2013. Reciprocal regulation of protein synthesis and carbon metabolism for thylakoid membrane biogenesis. *PLoS Biol* **11**, e1001482.

Bollenbach TJ, Stern DB, 2003. Divalent metal-dependent catalysis and cleavage specificity of CSP41, a chloroplast endoribonuclease belonging to the short chain dehydrogenase/reductase superfamily. *Nucleic Acids Res* **31**, 4317-25.

Brito Querido J, Diaz-Lopez I, Ramakrishnan V, 2024. The molecular basis of translation initiation and its regulation in eukaryotes. *Nat Rev Mol Cell Biol* **25**, 168-86.

Bruick RK, Mayfield SP, 1999. Light-activated translation of chloroplast mRNAs. *Trends Plant Sci* **4**, 190-5.

Calderon RH, De Vitry C, Wollman FA, Niyogi KK, 2023. Rubredoxin 1 promotes the proper folding of D1 and is not required for heme b(559) assembly in Chlamydomonas photosystem II. *J Biol Chem* **299**, 102968.

Calderon RH, Garcia-Cerdan JG, Malnoe A, *et al.*, 2013. A conserved rubredoxin is necessary for photosystem II accumulation in diverse oxygenic photoautotrophs. *J Biol Chem* **288**, 26688-96.

Cameron DL, Baber J, Shale C, *et al.*, 2021. GRIDSS2: comprehensive characterisation of somatic structural variation using single breakend variants and structural variant phasing. *Genome Biol* **22**, 202.

Cavaiuolo M, Kuras R, Wollman FA, Choquet Y, Vallon O, 2017. Small RNA profiling in Chlamydomonas: insights into chloroplast RNA metabolism. *Nucleic Acids Res* **45**, 10783-99.

Chidgey JW, Linhartova M, Komenda J, *et al.*, 2014. A cyanobacterial chlorophyll synthase-HliD complex associates with the Ycf39 protein and the YidC/Alb3 insertase. *Plant Cell* **26**, 1267-79.

Chotewutmontri P, Barkan A, 2020. Light-induced psbA translation in plants is triggered by photosystem II damage via an assembly-linked autoregulatory circuit. *Proc Natl Acad Sci U S A* **117**, 21775-84.

Chotewutmontri P, Williams-Carrier R, Barkan A, 2020. Exploring the Link between Photosystem II Assembly and Translation of the Chloroplast psbA mRNA. *Plants (Basel)* **9**.

Chua N-H, Gillham NW, 1977. The sites of synthesis of the principal thylakoid membrane polypeptides in Chlamydomonas reinhardtii. *The Journal of cell biology* **74**, 441-52.

Danon A, Mayfield SP, 1991. Light regulated translational activators: identification of chloroplast gene specific mRNA binding proteins. *EMBO J* **10**, 3993-4001.

Drapier D, Girard-Bascou J, Wollman FA, 1992. Evidence for Nuclear Control of the Expression of the atpA and atpB Chloroplast Genes in Chlamydomonas. *Plant Cell* **4**, 283-95.

Eberhard S, Loiselay C, Drapier D, *et al.*, 2011. Dual functions of the nucleus-encoded factor TDA1 in trapping and translation activation of atpA transcripts in Chlamydomonas reinhardtii chloroplasts. *Plant J* **67**, 1055-66.

Fauser F, Vilarrasa-Blasi J, Onishi M, *et al.*, 2022. Systematic characterization of gene function in the photosynthetic alga Chlamydomonas reinhardtii. *Nat Genet* **54**, 705-14.

Garcia-Cerdan JG, Furst AL, Mcdonald KL, Schunemann D, Francis MB, Niyogi KK, 2019. A thylakoid membrane-bound and redox-active rubredoxin (RBD1) functions in de novo assembly and repair of photosystem II. *Proc Natl Acad Sci U S A* **116**, 16631-40.

Genty B, Briantais JM, Baker NR, 1989. The Relationship between the Quantum Yield of Photosynthetic Electron-Transport and Quenching of Chlorophyll Fluorescence. *Biochim Biophys Acta* **990**, 87-92.

Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, 3rd, Smith HO, 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* **6**, 343-5.

Girard-Bascou J, Pierre Y, Drapier D, 1992. A nuclear mutation affects the synthesis of the chloroplast psbA gene production Chlamydomonas reinhardtii. *Curr Genet* **22**, 47-52.

Gohre V, Ossenbuhl F, Crevecoeur M, Eichacker LA, Rochaix JD, 2006. One of two alb3 proteins is essential for the assembly of the photosystems and for cell survival in Chlamydomonas. *Plant Cell* **18**, 1454-66.

Harris EH, Stern DB, Witman G, Harris EH, Harris EH, 2009. *The Chlamydomonas sourcebook*. Amsterdam ; Boston: Academic Press.

Heitzer M, Zschoernig B, 2007. Construction of modular tandem expression vectors for the green alga Chlamydomonas reinhardtii using the Cre/lox-system. *Biotechniques* **43**, 324, 6, 8 passim.

Hey D, Grimm B, 2018. ONE-HELIX PROTEIN2 (OHP2) Is Required for the Stability of OHP1 and Assembly Factor HCF244 and Is Functionally Linked to PSII Biogenesis. *Plant Physiol* **177**, 1453-72.

Jackson RJ, Hellen CU, Pestova TV, 2010. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat Rev Mol Cell Biol* **11**, 113-27.

Järvi S, Suorsa M, Aro EM, 2015. Photosystem II repair in plant chloroplasts--Regulation, assisting proteins and shared components with photosystem II biogenesis. *Biochim Biophys Acta* **1847**, 900-9.

Kafri M, Patena W, Martin L, *et al.*, 2023. Systematic identification and characterization of genes in the regulation and biogenesis of photosynthetic machinery. *Cell* **186**, 5638-55 e25.

Kavanagh KL, Jornvall H, Persson B, Oppermann U, 2008. Medium- and short-chain dehydrogenase/reductase gene and protein families : the SDR superfamily: functional and structural diversity within a family of metabolic and regulatory enzymes. *Cell Mol Life Sci* **65**, 3895-906.

Kim J, Mayfield SP, 1997. Protein disulfide isomerase as a regulator of chloroplast translational activation. *Science* **278**, 1954-7.

Kim J, Mayfield SP, 2002. The active site of the thioredoxin-like domain of chloroplast protein disulfide isomerase, RB60, catalyzes the redox-regulated binding of chloroplast poly(A)-binding protein, RB47, to the 5' untranslated region of psbA mRNA. *Plant Cell Physiol* **43**, 1238-43.

Knoppova J, Sobotka R, Tichy M, *et al.*, 2014. Discovery of a chlorophyll binding protein complex involved in the early steps of photosystem II assembly in Synechocystis. *Plant Cell* **26**, 1200-12.

Langmead B, Salzberg SL, 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods* **9**, 357-9.

Lefebvre-Legendre L, Choquet Y, Kuras R, Loubery S, Douchi D, Goldschmidt-Clermont M, 2015. A nucleus-encoded chloroplast protein regulated by iron availability governs expression of the photosystem I subunit PsaA in Chlamydomonas reinhardtii. *Plant Physiol* **167**, 1527-40.

Li X, Patena W, Fauser F, *et al.*, 2019a. A genome-wide algal mutant library and functional screen identifies genes required for eukaryotic photosynthesis. *Nat Genet* **51**, 627-35.

Li Y, Liu B, Zhang J, *et al.*, 2019b. OHP1, OHP2, and HCF244 Form a Transient Functional Complex with the Photosystem II Reaction Center. *Plant Physiol* **179**, 195-208.

Link S, Engelmann K, Meierhoff K, Westhoff P, 2012. The atypical short-chain dehydrogenases HCF173 and HCF244 are jointly involved in translational initiation of the psbA mRNA of Arabidopsis. *Plant Physiol* **160**, 2202-18.

Lu Y, 2016. Identification and Roles of Photosystem II Assembly, Stability, and Repair Factors in Arabidopsis. *Front Plant Sci* **7**, 168.

Mackinder LC, Meyer MT, Mettler-Altmann T, *et al.*, 2016. A repeat protein links Rubisco to form the eukaryotic carbon-concentrating organelle. *Proc Natl Acad Sci U S A* **113**, 5958-63.

Merchant SS, Prochnik SE, Vallon O, *et al.*, 2007. The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Science* **318**, 245-50.

Minai L, Wostrikoff K, Wollman FA, Choquet Y, 2006. Chloroplast biogenesis of photosystem II cores involves a series of assembly-controlled steps that regulate translation. *Plant Cell* **18**, 159-75.

Moummou H, Kallberg Y, Tonfack LB, Persson B, Van Der Rest B, 2012. The plant short-chain dehydrogenase (SDR) superfamily: genome-wide inventory and diversification patterns. *BMC Plant Biol* **12**, 219.

Mulo P, Sakurai I, Aro EM, 2012. Strategies for psbA gene expression in cyanobacteria, green algae and higher plants: from transcription to PSII repair. *Biochim Biophys Acta* **1817**, 247-57.

Myouga F, Takahashi K, Tanaka R, *et al.*, 2018. Stable Accumulation of Photosystem II Requires ONE-HELIX PROTEIN1 (OHP1) of the Light Harvesting-Like Family. *Plant Physiol* **176**, 2277-91.

Neusius D, Kleinknecht L, Teh JT, *et al.*, 2022. Lysine acetylation regulates moonlighting activity of the E2 subunit of the chloroplast pyruvate dehydrogenase complex in Chlamydomonas. *Plant J* **111**, 1780-800.

Nickelsen J, Rengstl B, 2013. Photosystem II assembly: from cyanobacteria to plants. *Annu Rev Plant Biol* **64**, 609-35.

Nixon PJ, Michoux F, Yu J, Boehm M, Komenda J, 2010. Recent advances in understanding the assembly and repair of photosystem II. *Ann Bot* **106**, 1-16.

O'connor BD, Van Der Auwera G, 2020. *Genomics in the Cloud: Using Docker, GATK, and WDL in Terra*. O'Reilly Media, Incorporated.

Oppermann UC, Filling C, Jornvall H, 2001. Forms and functions of human SDR enzymes. *Chem Biol Interact* **130-132**, 699-705.

Ossenbuhl F, Gohre V, Meurer J, Krieger-Liszkay A, Rochaix JD, Eichacker LA, 2004. Efficient assembly of photosystem II in Chlamydomonas reinhardtii requires Alb3.1p, a homolog of Arabidopsis ALBINO3. *Plant Cell* **16**, 1790-800.

Ossenbuhl F, Hartmann K, Nickelsen J, 2002. A chloroplast RNA binding protein from stromal thylakoid membranes specifically binds to the 5' untranslated region of the psbA mRNA. *Eur J Biochem* **269**, 3912-9.

Park S, Khamai P, Garcia-Cerdan JG, Melis A, 2007. REP27, a tetratricopeptide repeat nuclear-encoded and chloroplast-localized protein, functions in D1/32-kD reaction center protein turnover and photosystem II repair from photodamage. *Plant Physiol* **143**, 1547-60.

Picariello T, Hou Y, Kubo T, *et al.*, 2020. TIM, a targeted insertional mutagenesis method utilizing CRISPR/Cas9 in *Chlamydomonas reinhardtii*. *PLoS One* **15**, e0232594.

Porra R, Thompson W, Kriedemann P, 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* **975**, 384-94.

Rochaix JD, Kuchka M, Mayfield S, Schirmer-Rahire M, Girard-Bascou J, Bennoun P, 1989. Nuclear and chloroplast mutations affect the synthesis or stability of the chloroplast psbC gene product in Chlamydomonas reinhardtii. *EMBO J* **8**, 1013-21.

Schult K, Meierhoff K, Paradies S, Toller T, Wolff P, Westhoff P, 2007. The nuclear-encoded factor HCF173 is involved in the initiation of translation of the psbA mRNA in Arabidopsis thaliana. *Plant Cell* **19**, 1329-46.

Schwarz C, Elles I, Kortmann J, Piotrowski M, Nickelsen J, 2007. Synthesis of the D2 protein of photosystem II in Chlamydomonas is controlled by a high molecular mass complex containing the RNA stabilization factor Nac2 and the translational activator RBP40. *Plant Cell* **19**, 3627-39.

Shen JR, 2015. The Structure of Photosystem II and the Mechanism of Water Oxidation in Photosynthesis. *Annu Rev Plant Biol* **66**, 23-48.

Shen Y, Danon A, Christopher DA, 2001. RNA binding-proteins interact specifically with the Arabidopsis chloroplast psbA mRNA 5' untranslated region in a redox-dependent manner. *Plant Cell Physiol* **42**, 1071-8.

Somanchi A, Barnes D, Mayfield SP, 2005. A nuclear gene of Chlamydomonas reinhardtii, Tba1, encodes a putative oxidoreductase required for translation of the chloroplast psbA mRNA. *Plant J* **42**, 341-52.

Spaniol B, Lang J, Venn B, *et al.*, 2022. Complexome profiling on the Chlamydomonas lpa2 mutant reveals insights into PSII biogenesis and new PSII associated proteins. *J Exp Bot* **73**, 245-62.

Sueoka N, 1960. Mitotic Replication of Deoxyribonucleic Acid in Chlamydomonas Reinhardi. *Proc Natl Acad Sci U S A* **46**, 83-91.

Tokutsu R, Kato N, Bui KH, Ishikawa T, Minagawa J, 2012. Revisiting the supramolecular organization of photosystem II in Chlamydomonas reinhardtii. *J Biol Chem* **287**, 31574-81.

Vaistij FE, Boudreau E, Lemaire SD, Goldschmidt-Clermont M, Rochaix JD, 2000. Characterization of Mbb1, a nucleus-encoded tetratricopeptide-like repeat protein required for expression of the chloroplast psbB/psbT/psbH gene cluster in Chlamydomonas reinhardtii. *Proc Natl Acad Sci U S A* **97**, 14813-8.

Viola S, Cavaiuolo M, Drapier D, *et al.*, 2019. MDA1, a nucleus-encoded factor involved in the stabilization and processing of the atpA transcript in the chloroplast of Chlamydomonas. *Plant J* **98**, 1033-47.

Wakao S, Shih PM, Guan K, *et al.*, 2021. Discovery of photosynthesis genes through whole-genome sequencing of acetate-requiring mutants of *Chlamydomonas reinhardtii*. *PLoS Genet* **17**, e1009725.

Wang F, Dischinger K, Westrich LD, *et al.*, 2023a. One-helix protein 2 is not required for the synthesis of photosystem II subunit D1 in Chlamydomonas. *Plant Physiology* **191**, 1612-33.

Wang F, Johnson X, Cavaiuolo M, Bohne AV, Nickelsen J, Vallon O, 2015. Two Chlamydomonas OPR proteins stabilize chloroplast mRNAs encoding small subunits of photosystem II and cytochrome b6 f. *Plant J* **82**, 861-73.

Wang L, Patena W, Van Baalen KA, *et al.*, 2023b. A chloroplast protein atlas reveals punctate structures and spatial organization of biosynthetic pathways. *Cell* **186**, 3499-518 e14.

Wang X, Gao F, Zhang J, Zhao J, Ogawa T, Ma W, 2016. A Cytoplasmic Protein Ssl3829 Is Important for NDH-1 Hydrophilic Arm Assembly in Synechocystis sp. Strain PCC 6803. *Plant Physiol* **171**, 864-77.

Wollman F-A, Minai L, Nechushtai R, 1999. The biogenesis and assembly of photosynthetic proteins in thylakoid membranes1This article is dedicated to the memory of our colleague and friend, Alma Gal. Her presence played a major role in our decision to prepare this review article.1. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1411**, 21-85.

Yohn CB, Cohen A, Danon A, Mayfield SP, 1996. Altered mRNA binding activity and decreased translational initiation in a nuclear mutant lacking translation of the chloroplast psbA mRNA. *Mol Cell Biol* **16**, 3560-6.

Yohn CB, Cohen A, Danon A, Mayfield SP, 1998a. A poly(A) binding protein functions in the chloroplast as a message-specific translation factor. *Proc Natl Acad Sci U S A* **95**, 2238-43.

Yohn CB, Cohen A, Rosch C, Kuchka MR, Mayfield SP, 1998b. Translation of the chloroplast psbA mRNA requires the nuclear-encoded poly(A)-binding protein, RB47. *J Cell Biol* **142**, 435-42.

Zhang R, Patena W, Armbruster U, Gang SS, Blum SR, Jonikas MC, 2014. High-Throughput Genotyping of Green Algal Mutants Reveals Random Distribution of Mutagenic Insertion Sites and Endonucleolytic Cleavage of Transforming DNA. *Plant Cell* **26**, 1398-409.

Table 1

Table 1. Chlorophyll content and Chl a/b ratios in WT and Δ*CrHCF244-T23*. Cells from mixotrophic cultures in the early exponential phase were used for measurement.

	WT	**Δ*CrHCF244-T23***
[Chl]	38.9 ± 2 μg mL^{-1} OD_{750}^{-1}	40.7 ± 2.5 μg mL^{-1} OD_{750}^{-1}
Chl a/b	2.54 ± 0.06	2.27 ± 0.07

Figure legends:

Figure 1 ami6 growth, PSII activity, and the mapping of genomic variation and the causative gene.

(A) Growth of wild type (WT), *ami6* mutant and two complemented lines of *ami6*, under mixotrophic (TAP) and photoautotrophic (HS) conditions. *ami6-C1* is a representative transformant from transforming *ami6* with the C1 construct (illustrated in **(C)**); *ami6-C2* is a representative transformant from transforming *ami6* with the C2 construct (illustrated in **(C)**). F_v/F_m values are shown for cultures grown on TAP. **(B)** Mapping of *ami6* genomic variation by whole genome sequencing revealed a ~347 kb deletion on chromosome 15. **(C)** Illustration of genetic constructs for complementing *ami6* mutant. The C1 and C2 constructs both are under control of the *psaD* promoter (*PpsaD*). The constructs were used for transforming untagged CrHCF244 and Venus-tagged CrHCF244 into the *ami6* mutant.

Figure 2 Growth and PSII activity of a CRISPR-Cas9 knockout CrHCF244 mutant.

(A) *CrHCF244* gene map (5' UTR as green box, exons as blue boxes, 3' UTR as purple box) indicating the gRNA targeting site at exon 2 for CRISPR-Cas9 knockout/gene editing. **(B)** Growth of WT, CRISPR-Cas9 knockout *CrHCF244* mutant Δ*CrHCF244-T23* (shown as *T23* in **(B)** and **(C)**) and the two complemented strains (shown as *T23-C1* and *T23-C2* in **(B)** and **(C)**), which are representative transformants from transforming Δ*CrHCF244-T23* with C1 and C2 construct (illustrated in Fig. 1C), respectively, under mixotrophic (TAP) and photoautotrophic (HS) conditions. **(C)** F_v/F_m values for strains grown on TAP.

Figure 3 Accumulation of photosynthetic protein complexes.

(A) Immunoblots of photosynthetic proteins in WT, *ami6* and its two complemented lines (*ami6-C1*, *ami6-C2*), Δ*CrHCF244-T23* and its two complemented lines (*T23-C1*, *T23-C2*) and Fud7 (*psbA* deletion mutant). **(B)** Blue-Native PAGE (BN-PAGE) separation of photosynthetic protein complexes. Arrows indicate the positions of different PSII complexes. **(C)** Immunoblotting analysis using D1 antibody of the PSII complexes. The immunoblotting was performed after transferring the protein complexes on the BN-PAGE to PVDF membrane. Arrows indicate PSII complexes, I and II: PSII super-complexes, III: PSII dimer, IV: PSII monomer.

Figure 4 D1 synthesis and psbA transcription.

(A) ^{14}C-acetate pulse-labeling of WT, *ami6* mutant, Fud7 (*psbA* deletion) and CRISPR-Cas9 knockout mutant Δ*CrHCF244-T23*. Cells were labeled for 20 min in the presence of 10 µg/mL cycloheximide to inhibit cytosol translation. PSII major subunits are shown by the arrows. **(B)** Quantitative real time PCR (qRT-PCR) to detect *psbA* transcription change in WT and *ami6* mutant. Three pairs of *psbA* primers were used for the qRT-PCR. Names of primers were shown here. The sequences of primers are shown in Table S1.

Figure 5 Complementation of Chlamydomonas ami6 mutant with the Arabidopsis HCF244 gene.

(A) Schematic of the construct for transforming *ami6* with the *Arabidopsis HCF244* gene. Mature AtHCF244 protein (mAtHCF244) in fusion with a chloroplast transit peptide (cTP) derived from PsaD in *Chlamydomonas* was expressed under control of *psaD* promoter (*PpsaD*) **(B)** Growth and PSII activity of WT, *ami6* mutant, and the two complemented strains under mixotrophic (TAP) and photoautotrophic (HS) conditions. F_v/F_m values are shown for strains grown on TAP.

Figure 6 Chloroplast localization of CrHCF244.

Venus-tagged CrHCF244 plasmid was transformed into the *ami6* mutant. The complemented line *ami6-C2* was grown under photosynthetic condition before imaging.

Figure 7 Suppressor mutants of Δ*CrHCF244-T23* suggest an alternative pathway for D1 translation control.

(A) Growth and PSII activity of strains under mixotrophic conditions. WT, CRISPR-Cas9 knockout mutant Δ*CrHCF244-T23* (labeled as *T23* here) and three suppressor mutants (labeled as *T23-Su1*, *T23-Su2*, *T23-Su3*). **(B)** Proposed working model of alternative *psbA* translation control. In the absence of CrHCF244, the ribosome cannot bind *psbA* mRNA thus there is no D1 translation in the Δ*CrHCF244-T23* mutant. A mutation in a regulator gene actives an alternative translation factor (TF), which allows ribosome loading to *psbA* mRNA, thus enabling D1 translation in the suppressor mutants.

Figure 8 Growth and PSII activity of RB47 and RB60 mutants.

(A) Gene maps of the *RB47* and *RB60* showing the CRISPR-Cas9 cleavage sites. **(B)** Growth of the *RB47* mutant (Δ*RB47*) and RB60 (Δ*RB60-1*, Δ*RB60-2*, Δ*RB60-3*) mutants generated by CRISPR-Cas9, under photoautotrophic conditions. PSII activity was measured as F_v/F_m. **(C)** Abundance of D1 subunit in the mutants. Immunoblotting of D1 subunit and RB60 was performed in the strains; Coomassie brilliant blue (CBB) stain was shown as a loading control.

Figures

Figure 1

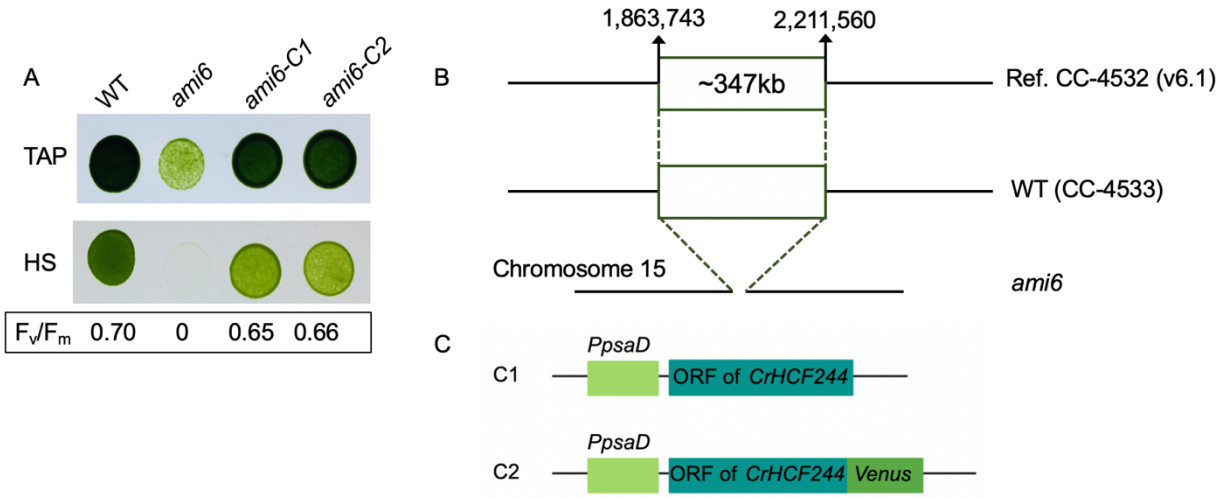
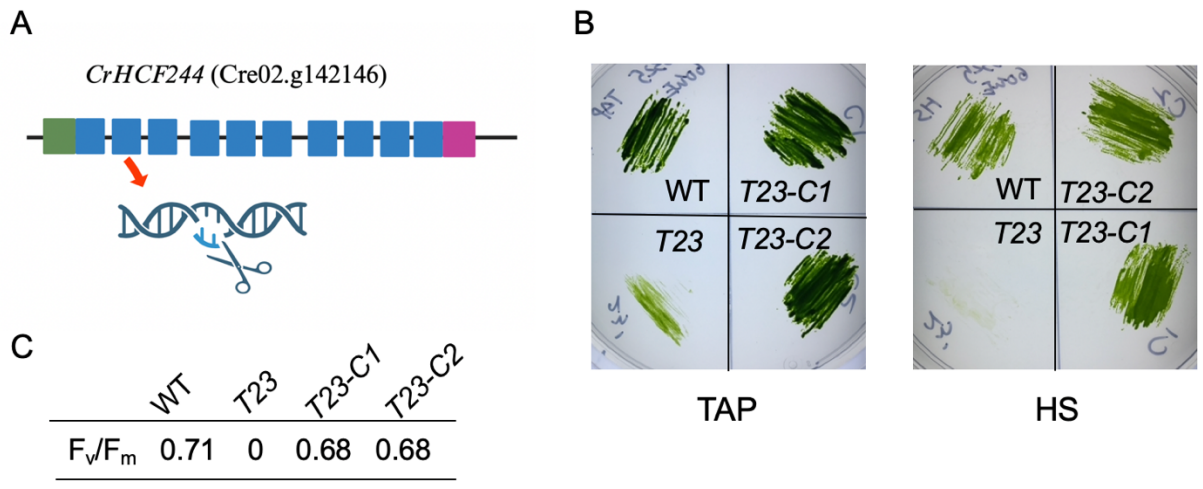


Figure 1 ami6 growth, PSII activity, and the mapping of genomic variation and the causative gene.

(A) Growth of wild type (WT), *ami6* mutant and two complemented lines of *ami6*, under mixotrophic (TAP) and photoautotrophic (HS) conditions. *ami6-C1* is a representative transformant from transforming *ami6* with the C1 construct (illustrated in **(C)**); *ami6-C2* is a representative transformant from transforming *ami6* with the C2 construct (illustrated in **(C)**). F_v/F_m values are shown for cultures grown on TAP. **(B)** Mapping of *ami6* genomic variation by whole genome sequencing revealed a ~347 kb deletion on chromosome 15. **(C)** Illustration of genetic constructs for complementing *ami6* mutant. The C1 and C2 constructs both are under control of the *psaD* promoter (*PpsaD*). The constructs were used for transforming untagged CrHCF244 and Venus-tagged CrHCF244 into the *ami6* mutant.

Figure 2



	WT	T23	T23-C1	T23-C2
F_v/F_m	0.71	0	0.68	0.68

Figure 2 Growth and PSII activity of a CRISPR-Cas9 knockout CrHCF244 mutant.

(A) *CrHCF244* gene map (5' UTR as green box, exons as blue boxes, 3' UTR as purple box) indicating the gRNA targeting site at exon 2 for CRISPR-Cas9 knockout/gene editing. **(B)** Growth of WT, CRISPR-Cas9 knockout *CrHCF244* mutant Δ*CrHCF244-T23* (shown as *T23* in **(B)** and **(C)**) and the two complemented strains (shown as *T23-C1* and *T23-C2* in **(B)** and **(C)**), which are representative transformants from transforming Δ*CrHCF244-T23* with C1 and C2 construct (illustrated in Fig. 1C), respectively, under mixotrophic (TAP) and photoautotrophic (HS) conditions. **(C)** F_v/F_m values for strains grown on TAP.

Figure 3

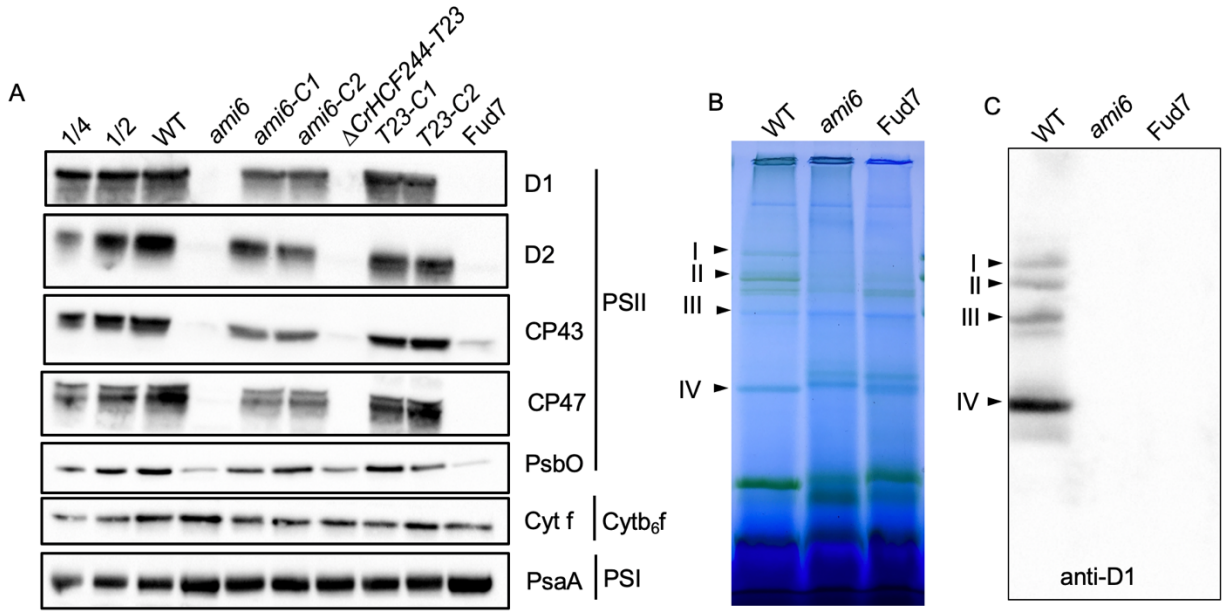


Figure 3 Accumulation of photosynthetic protein complexes.

(A) Immunoblots of photosynthetic proteins in WT, *ami6* and its two complemented lines (*ami6-C1*, *ami6-C2*), Δ*CrHCF244-T23* and its two complemented lines (*T23-C1*, *T23-C2*) and Fud7 (*psbA* deletion mutant). **(B)** Blue-Native PAGE (BN-PAGE) separation of photosynthetic protein complexes. Arrows indicate the positions of different PSII complexes. **(C)** Immunoblotting analysis using D1 antibody of the PSII complexes. The immunoblotting was performed after transferring the protein complexes on the BN-PAGE to PVDF membrane. Arrows indicate PSII complexes, I and II: PSII super-complexes, III: PSII dimer, IV: PSII monomer.

Figure 4

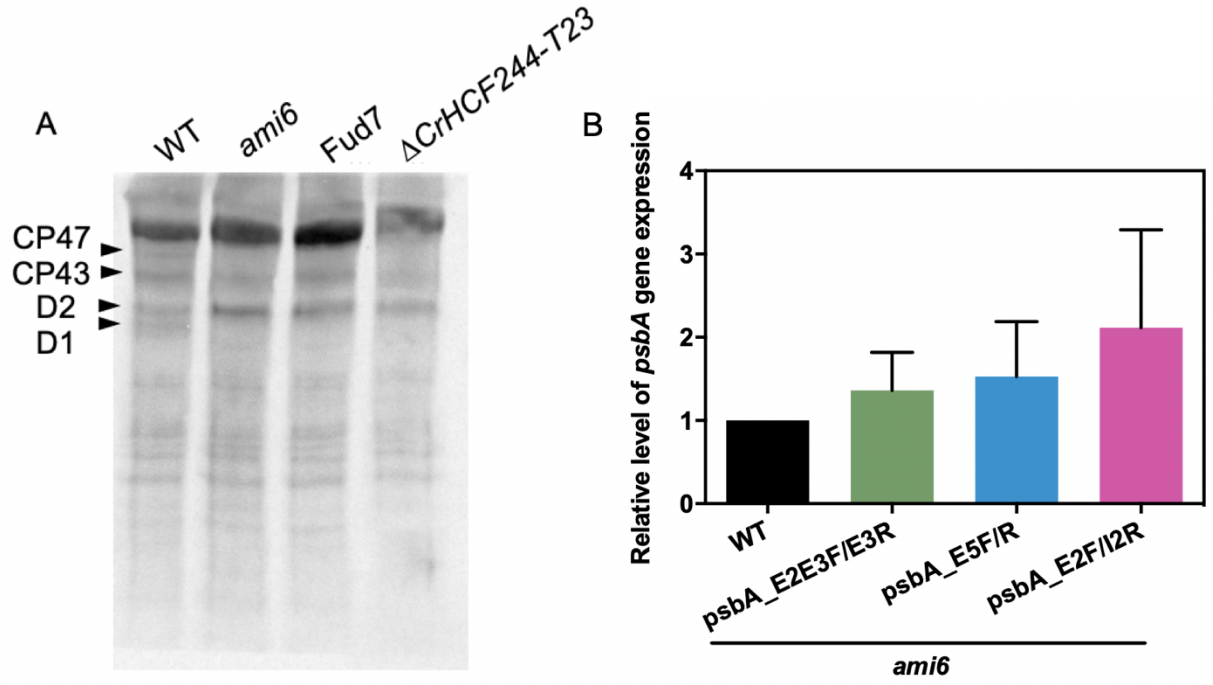


Figure 4 D1 synthesis and psbA transcription.

(A) ^{14}C-acetate pulse-labeling of WT, *ami6* mutant, Fud7 (*psbA* deletion) and CRISPR-Cas9 knockout mutant Δ*CrHCF244-T23*. Cells were labeled for 20 min in the presence of 10 µg/mL cycloheximide to inhibit cytosol translation. PSII major subunits are shown by the arrows. **(B)** Quantitative real time PCR (qRT-PCR) to detect *psbA* transcription change in WT and *ami6* mutant. Three pairs of *psbA* primers were used for the qRT-PCR. Relative expression from each pair of primers in WT was set to 1.0. Names of primers were shown here. The sequences of primers are shown in Table S1.

Figure 5

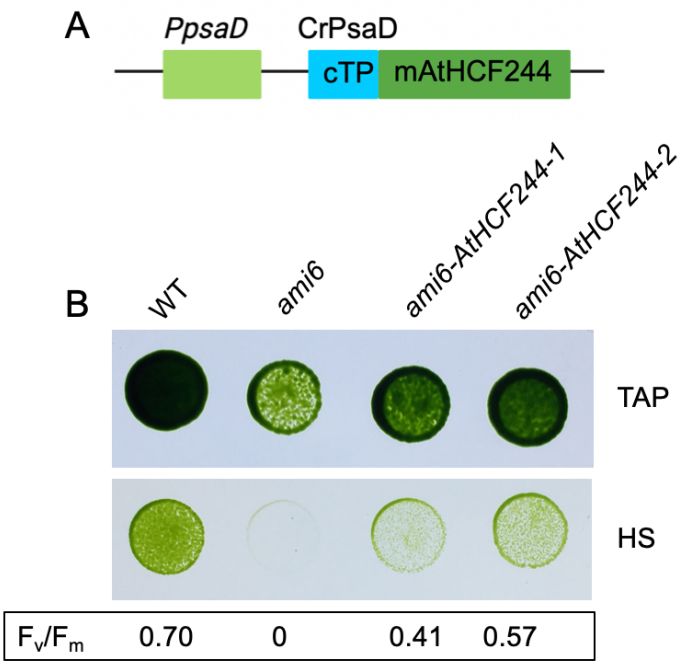


Figure 5 Complementation of Chlamydomonas ami6 mutant with the Arabidopsis HCF244 gene.

(A) Schematic of the construct for transforming *ami6* with the *Arabidopsis HCF244* gene. Mature AtHCF244 protein (mAtHCF244) in fusion with a chloroplast transit peptide (cTP) derived from PsaD in *Chlamydomonas* was expressed under control of *psaD* promoter (*PpsaD*) **(B)** Growth and PSII activity of WT, *ami6* mutant, and the two complemented strains under mixotrophic (TAP) and photoautotrophic (HS) conditions. F_v/F_m values are shown for strains grown on TAP.

Figure 6

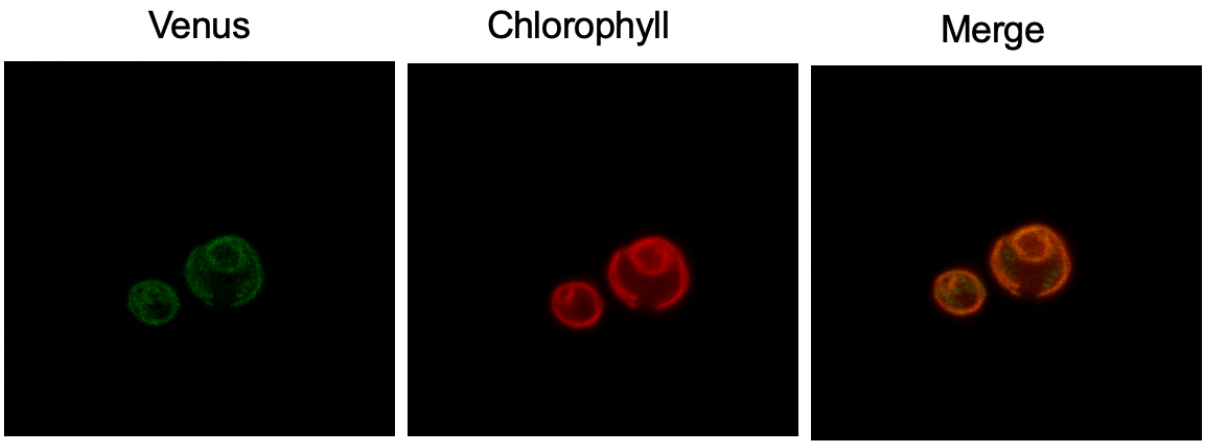


Figure 6 Chloroplast localization of CrHCF244.

Venus-tagged CrHCF244 plasmid was transformed into the *ami6* mutant. The complemented line *ami6-C2* was grown under photosynthetic condition before imaging.

Figure 7

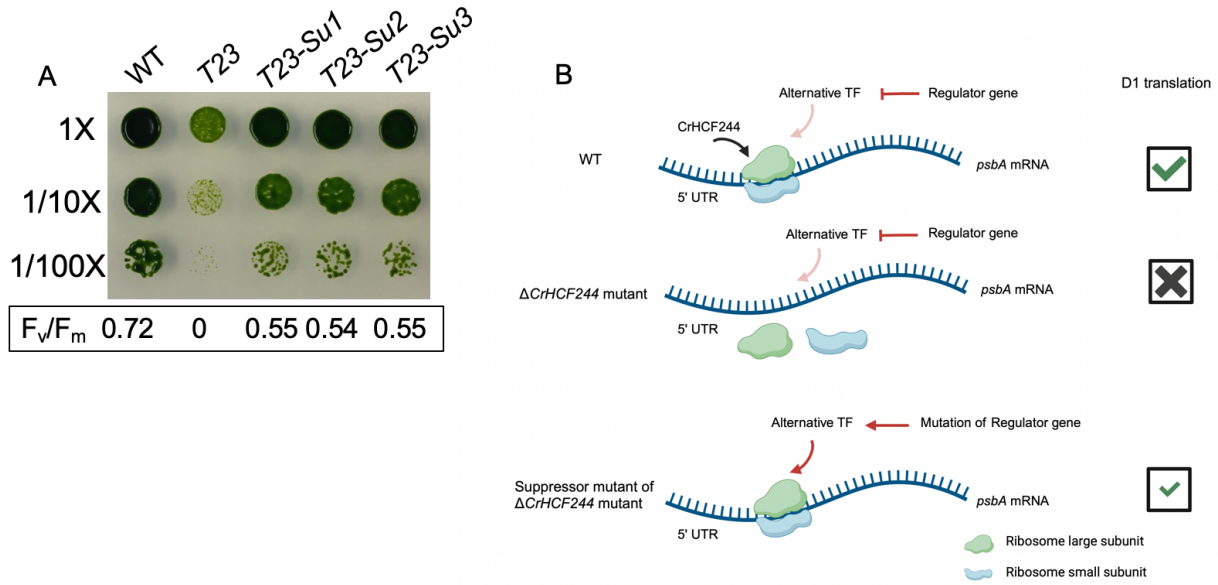


Figure 7 Suppressor mutants of ΔCrHCF244-T23 suggest an alternative pathway for D1 translation control.

(A) Growth and PSII activity of strains under mixotrophic conditions. WT, CRISPR-Cas9 knockout mutant Δ*CrHCF244-T23* (labeled as *T23* here) and three suppressor mutants (labeled as *T23-Su1*, *T23-Su2*, *T23-Su3*). **(B)** Proposed working model of alternative *psbA* translation control. In the absence of CrHCF244, the ribosome cannot bind *psbA* mRNA thus there is no D1 translation in the Δ*CrHCF244-T23* mutant. A mutation in a regulator gene actives an alternative translation factor (TF), which allows ribosome loading to *psbA* mRNA, thus enabling D1 translation in the suppressor mutants.

Figure 8

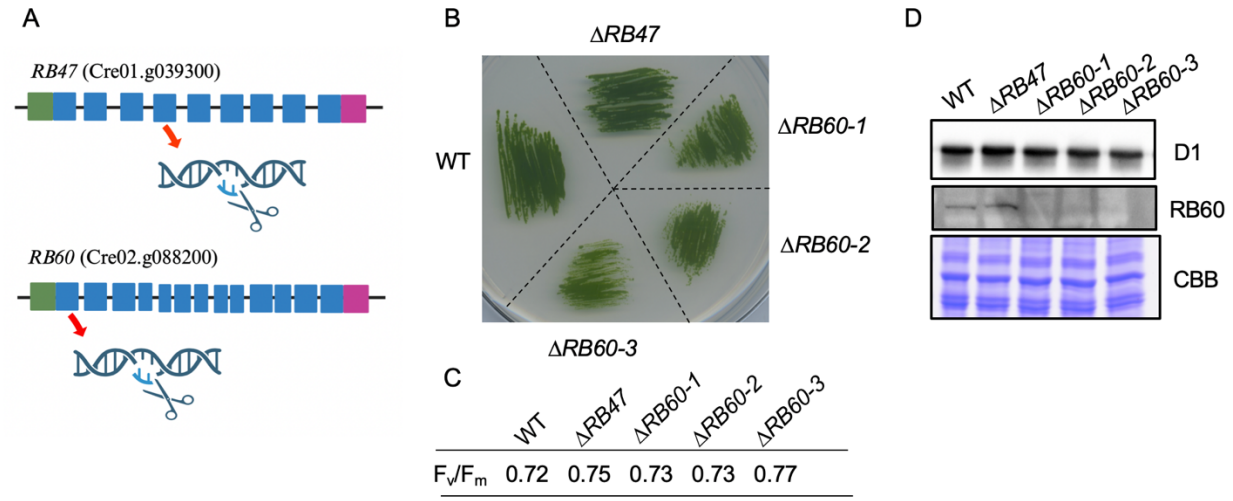


Figure 8 Growth and PSII activity of RB47 and RB60 mutants.

(A) Gene maps of the *RB47* and *RB60* showing the CRISPR-Cas9 cleavage sites. **(B)** Growth of the *RB47* mutant (Δ*RB47*) and RB60 (Δ*RB60-1*, Δ*RB60-2*, Δ*RB60-3*) mutants generated by CRISPR-Cas9, under photoautotrophic conditions. PSII activity was measured as F_v/F_m. **(C)** Abundance of D1 subunit in the mutants. Immunoblotting of D1 subunit and RB60 was performed in the strains; Coomassie brilliant blue (CBB) stain was shown as a loading control.