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Essential Histidine and Tryptophan Residues in CcsA, a System II Polytopic Cytochrome *c* Biogenesis Protein*[S]

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Three distinct systems (I, II, and III) for catalysis of heme attachment to *c*-type apocytochromes are known. The CcsA and Ccs1 proteins are required in system II for the assembly of bacterial and plastid cytochromes *c*. A tryptophan-rich signature motif (WWD), also occurring in CcmC and CcmF found in system I, and three histidinyl residues, all strictly conserved in CcsA suggest a function in heme handling. Topological analysis of plastid CcsA in bacteria using the *PhoA* and *LacZα* reporters placed the WWD motif, the conserved residues His^{212} and His^{347} on the lumen side of the membrane, whereas His^{309} was assigned a location on the stromal side. Functional analysis of CcsA through site-directed mutagenesis enabled the designation of the initiation codon of the *ccsA* gene and established the functional importance of the WWD signature motif and the absolute requirement of all three histidines for the assembly of plastid *c*-type cytochromes. In a *ccsA* mutant, a 200-kDa Ccs1-containing complex is absent from solubilized thylakoid membranes, suggesting that CcsA operates together with Ccs1. We propose a model where the WWD motif and histidine residues function in relaying heme from stroma to lumen and we postulate the existence of a cytochrome *c* assembly machinery containing CcsA, Ccs1 and additional components.

The *c*-type cytochromes are ubiquitous metalloproteins which reside on the *p*-side of energy transducing membrane systems. They display very little sequence similarity apart from the occurrence of one to four conserved C*XX*CH motifs which are the binding sites for the prosthetic group. The *c*-type cytochromes are distinguished from other cytochromes by covalent attachment of the prosthetic group to the apoprotein via one, or in most cases, two thioether linkages between the vinyl side chains of heme and the cysteines from the C*XX*CH motif. Because all holocytochromes *c* acquire their function in a different compartment from the sites of synthesis of their apoproteins and cofactor heme, their biogenesis follows a complex pathway with several steps: (*a*) transport and processing of the apocytochromes, (*b*) transport of heme, (*c*) transfer of reducing equivalents across the membrane in order to assure the maintenance of heme and sulfhydryl groups of the C*XX*CH motif under a reduced state and d) stereospecific attachment of the heme group to the apocytochromes. Remarkably, genetic and biochemical analyses of *c*-type cytochromes biogenesis have demonstrated the existence of three distinct systems for the maturation of these molecules (for review 1, 2–6).

The first pathway to be defined, now referred to as system III (3), was discovered originally in fungal mitochondria and is also believed to operate in vertebrate and invertebrate mitochondria. System III appears to be the least complex of the three systems as only two factors, cytochrome *c* heme lyase and cytochrome c_1 heme lyase were found to be required for the assembly of the two *c*-type cytochromes found in the mitochondrial intermembrane space, cyt[1] *c* and cyt c_1, respectively (7–10). The interaction of cytochrome *c* heme lyase with both heme (11), via the characteristic CPX heme binding motif (12), and apocytochrome (13) led to the assumption that it functions in the catalysis of thioether bond formation (7, 14–16).

A contrasting situation occurs in system I which operates in α and γ-proteobacteria, Deinococcus, archaea, plant and protozoal mitochondria. Extensive functional studies in *Rhodobacter capsulatus* (17–20), *Paracoccus denitrificans* (21–24) and *Escherichia coli* (25, 26) microbial models have led to the identification of as many as 12 genes whose products are recruited to complete holocytochrome *c* maturation (for review 2, 27, 28). A detailed view of this pathway has now emerged on the basis of thorough biochemical investigation of the different gene products. A complex of multiple membrane and periplasmic proteins proposed to catalyze heme transport across the membrane (17, 18, 22, 29–32), the transfer of reducing equivalents (20, 23, 33–37), the reduction and chaperoning of apocytochromes (19, 27, 38–41), the handling and ligation of heme onto the apocytochromes (26, 28, 42–46) is now envisioned to function as an apparatus for *c*-type cytochrome assembly.

System II was discovered in the green alga *Chlamydomonas reinhardtii* in a search for *ccs* mutants specifically deficient in membrane-bound cyt *f* and soluble cyt c_6, the only two *c*-type cytochromes resident in the plastid (47). All the *ccs* mutants failed to accumulate the plastid holocytochromes *c* because of compromised heme attachment in the lumen (47, 48). Genetic analysis established that five nuclear loci (*CCS1* to *5*) and one plastid locus (*ccsA*) are required for the biogenesis of plastid *c*-type cytochromes (47, 49, 50). At present, only *ccsA* (51) and *CCS1* (52) have been characterized at the molecular level and

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[1] The abbreviations used are: cyt, cytochrome; BN, blue native; ccs, cytochrome *c* synthesis; Chl, chlorophyll; ORF, open reading frame; PVDF, polyvinylidene difluoride; $Spec^R$, spectinomycin-resistant; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

shown to encode previously unknown integral membrane proteins, for which function can only be inferred based on our knowledge of the biochemistry required to complete holocytochrome *c* maturation. Ccs1 is a pioneer protein lacking any recognizable motifs which might suggest an obvious biochemical activity in the pathway (52). CcsA, on the other hand, has a tryptophan-rich[2] "WWD" motif (WGXϕWXWD, where ϕ is an aromatic residue), similar to that found in the CcmC and CcmF proteins involved in the biogenesis of *c*-type cytochromes in system I (17, 25, 28, 31, 53). The presence of strictly conserved histidinyl residues which are known ferroheme ligands led to the hypothesis that CcmC, CcmF and CcsA proteins bind and deliver heme to the apocytochromes (6, 31, 51). However, despite the conservation of histidinyl and tryptophan residues, CcsA is not a true homolog of CcmC or CcmF and clearly defines a separate member of the WWD motif family (5, 31). The co-occurrence of Ccs1 and CcsA homologues, encoded by genes often organized in an operon arrangement, in plastid and bacterial genomes led to the definition of a new pathway for assembling holocytochromes *c* (3, 5, 6). In *Bacillus subtilis*, *Synechocystis* sp. and *Bordetella pertussis*, functional genomics and classical genetics have also implicated the CcsA and Ccs1 homologues in the assembly of *c*-type cytochromes (54–58) but a clear function in the assembly process has not yet been assigned to either of these two proteins.

In an effort to elucidate the biochemical function of CcsA in the plastid system, we used the bacterial topological reporters alkaline phosphatase and β-galactosidase to deduce the polytopic arrangement of *Chlamydomonas* CcsA and thus to determine the orientation of the WWD motif and conserved histidine residues. The functional significance of these key residues was further demonstrated by site-directed mutagenesis of the *ccsA* gene in the plastid context and each mutant was assessed for its ability to assemble the *c*-type cytochromes and support photosynthetic growth. We also provide the first evidence for the existence of a system II cytochrome assembly machinery by showing that CcsA and Ccs1 are present in a 200-kDa complex that could be detected within the thylakoid membranes by BN-PAGE. Based on our results, we formulate and discuss the hypothesis that CcsA and Ccs1 constitute a subcomplex functioning in the delivery/ligation of heme to apocytochromes.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—C. reinhardtii wild type strains CC125, 2137 (*Chlamydomonas* Genetic Center, Duke University) and WT59$^+$ (Institut de Biologie PhysicoChimique, Paris, France) are wild type with respect to photosynthesis and *c*-type cytochrome biogenesis and are accordingly labeled *wild type* in the figures. The *ccsA*-ct59, -B6 (49) and *ccs1–6*::*ARG7* (59) strains display a "ccs" phenotype (47, 48) and are labeled accordingly *ccsA* and *ccs1* in the figures. Strains were grown at 22–25 °C in TAP medium (60) with or without copper supplementation under dim light (25 μmol/m^2/s) for non photosynthetic strains or under standard illumination for wild type strains (125 μmol/m^2/s) as described previously (47). The *ccsA* site-directed mutants were tested for their ability to grow photosynthetically on minimum medium under various illuminations (40–170-500 μmol/m^2/s). *E. coli* strains XL1-blue and DH5α were used as hosts for recombinant DNA techniques (61). The Lac$^-$ Pho$^-$ strain CC118 (62) was used for alkaline phosphatase assays and DH5α for β-galactosidase assays.

Construction of ccsA-PhoA and ccsA-LacZ Fusions—Plasmid pRGK200 (19) containing the alkaline phosphatase encoding gene (*phoA*) was used to generate 9 *ccsA-phoA* translational fusions by a PCR-based strategy described previously (17). Various portions of the *ccsA* gene were amplified with *Pfu* polymerase using the cloned *ccsA* gene as a template, F-CcsASac as a forward primer and one of the 9 R-CcsA oligonucleotides as a reverse primer (see list of primers below). F-CcsASac was engineered with a *Sac*I site upstream of the first ATG codon in *ccsA* ORF (51) and reverse primers were designed with a *Sal*I site at the desired *ccsA-phoA* fusion junction. PCR products thus obtained were digested with *Sac*I and *Sal*I and cloned into *Sac*I/*Sal*I digested pRGK200 to yield the p*ccsA*::*phoA* series of plasmids. The p*ccsA*::*phoA* plasmids express translational fusions of *ccsA* to *phoA* with fusions at positions 14, 65, 138, 168, 200, 247, 288, 321, 352 of CcsA polypeptide. The *ccsA*::*LacZ*α fusions at positions 14, 247, and 321 were generated from the corresponding p*ccsA*::*phoA* plasmids by replacing a 2.6-kb *Sal*I-*Pst*I fragment including the entire *phoA* gene with a 0.7-kb PCR-amplified *lacZ* segment corresponding to the α fragment of β-galactosidase and in frame with the upstream CcsA reading frame. The *lacZ*α segment was amplified from plasmid pSK$_{II}^+$ (Stratagene) with M13 reverse primer and a *lacZ* specific primer engineered with a *Pst*I site. The PCR product was digested with *Sal*I and *Pst*I and then cloned into *Pst*I-*Sal*I digested p*ccsA*::*phoA* plasmids.

F-CcsASac, 5′-CGCGACTCTATGAATTTTGTTAATTTAG-3′;
R-CcsA14, 5′-ACGCGTCGACCGTAAAGAATTTTCAATTT-3′;
R-CcsA65, 5′-ACGCGTCGACTTGTTTGCTTGAAACCCGTC-3′;
R-CcsA138, 5′-ACGCGTCGACTTGTTTGCTTGAAACCCGTC-3′;
R-CcsA168, 5′-ACGCGTCGACTCAACCAATAAAGTAAAACTTG-3′;
R-CcsA200, 5′-ACGCGTCGACAATGGTGATGCTTGTTGC-3′;
R-CcsA247, 5′-ACGCGTCGACTCATACTTTTGGGTGTACC-3′;
R-CcsA288, 5′-ACGCGTCGACCTCCAATATGATCCCCATGC-3′;
R-CcsA321, 5′-ACGCGTCGACGGTTTTTCACCTTCCCAACC-3;
R-CcsA352, 5′-ACGCGTCGACTTAAAAAAACCATAACTATG-3′.

Measurement of PhoA and LacZ Activities—Alkaline phosphatase assays were performed on whole *E. coli* cells as described previously in (63), except that isopropyl-1-thio-β-D-galactopyranoside was not used to induce the expression of the fusion proteins. β-galactosidase activity was measured on SDS-chloroform treated cells essentially as described in (64). Alkaline phosphatase activity expressed in Miller units was calculated using the following formula: $(1000 \cdot A_{420\ nm})/(\text{time (min)} \cdot A_{600\ nm})$. β-Galactosidase activity is expressed in units and calculated by the following formula: $(1000 \cdot (A_{420\ nm} - (1.75 \cdot A_{550\ nm}))/(\text{time (min)} \cdot A_{600\ nm})$.

Site-directed Mutagenesis of ccsA—Site-directed mutants of CcsA: $ATG_1 \rightarrow GAG$, $ATG_2 \rightarrow GCG$, H212D, H309E, H347E, H347A, W279A, W284A, W288A, W290A, W288AW290A, W296A, W301A, D291A, D291E, and D291N were constructed by a PCR approach or with the Sculptor *in vitro* mutagenesis kit (Amersham Biosciences) using as a template plasmid pEBP or M13mp18 vector containing the *ccsA* gene (51) and the set of primers listed in Table I. Mutagenic primers were designed in order to introduce a new restriction site or to alter an existing one in the sequence of the *ccsA* gene (see Table I). All PCR amplified regions of *ccsA* were sequenced on both strands. Plasmid pPmP-*aadA* was obtained by cloning a 5.5-kb *Pme*I-*Pst*I chloroplast DNA containing the *ccsA* gene into the *Xba*I (Klenow-filled) and *Nsi*I-digested pGEM7Z plasmid (Promega). To simplify cloning, the *Hin*dIII site, located at the 3′ far end of the *ccsA* gene was removed by site-directed mutagenesis. An *Eco*RI-*Sma*I fragment containing the *aadA* gene expression cassette from the plasmid pUC-atpX-aad (65) was cloned into the *Mlu*I (Klenow-treated) and *Eco*RI digested pPmP vector to yield pPmP-*aadA*. Mutations H212D, H309E, H347E, H347A, W279A, W284A, W288A, W290A, W288AW290A, D291A, D291E, and D291N in *ccsA* were transferred to pPmP-*aadA* as a 1.9-kb *Eco*RI-*Hin*dIII or a 1.7-kb *Eco*RI-*Pac*I fragment. Plasmid $pATG_1 \rightarrow GAG$ was co-transformed with plasmid p228 carrying a mutant allele of the 16 S rRNA gene which confers resistance to spectinomycin (66).

Transformation of the Chloroplast Genome—C. reinhardtii vegetative cells grown in TAP liquid culture (1–2 $\times$ 10^6 cells/ml) were transformed by particle bombardment (using a home-made particle gun at Institut de Biologie PhysicoChimique, Paris or a Bio-Rad PDS-1000/He particle delivery system at UCLA) as described in (51). Particles were coated with DNA as in (67). We also found in the course of this study that the transformation of gametes significantly facilitates the recovery of homoplasmic strains carrying a site-directed mutation in *ccsA* (see section below). Gamete cells for transformation were prepared as follows. Vegetative cells grown in liquid TAP medium (0.5 $\times$ 10^6 cells/ml) were collected by centrifugation, washed once with sterile water and resuspended in TAP without nitrogen source in order to induce gametogenesis (60). Cells were maintained in the dark with agitation for 16–20 h, then centrifuged, washed once in water and resuspended in 50 mM sorbitol (10^7 cells/ml). 5 $\times$ 10^6 to 10^7 cells were plated on agar and immediately bombarded. Bombarded cells were incubated overnight under dim light. For the complementation experiments, *ccsA*-B6 or *ccsA*-ct59 cells were bombarded directly on solid minimal agar. Bombarded cells were then incubated at a higher light intensity (50 μmol/m^2/s). For the generation of Spec^R *ccsA* site-directed mutants, wild type

[2] The signature motif "WG*X*ϕW*X*WD" will be referred to as WWD motif throughout the article.

TABLE I
Primers used for ccsA site-directed mutagenesis

The boldface lowercase letters indicate the nucleotide change in the mutagenic primers that was designed in order to gain or lose (Δ) a restriction site in the sequence of the *ccsA* gene (modification).

Primers	Sequence (5′–3′)	Modification
15–4	CCATTAAGTAATTTATA	
15–9	ACCATAACTATGTAAACC	
15–11	TATGGCATGTAATACTCC	
15–13	CAGGCAACTACTCCTTCGG	
15–14	CAAGAATGTCAATCTCC	
ATG_1	CAAAATTCT**cg**AGAAATATATTCG	*Xho*I
ATG_2	CTAAAAAAAGC**gc**GCAAAAAGTAGC	*Bss*HII
H212D	CATTACAGT**g**AC**g**TcCATCATTAACC	Δ*Nsi*I
H309E	GTTAAACGGGT**cTcg**AGATAAATAGC	*Xho*I
H347E	CCATAACT**cTcgAg**ACCTTCTCC	*Xho*I
H347A	CCATA**g**CTA**gc**TAAACCTTCTACC	*Nhe*I
W279A-2	CCATGCTTCATTAGC**agc**CAC**t**GCCCCAGATAAAA	*Alw*NI
W284A-1	CCAATATGATCC**tgca**GCTTCATTAGCC	*Pst*I
W284A-2	GGCTAATGAAGC**tgca**GGATCATATTGG	*Pst*I
W288A	GTTTCTTTTGGATCCCAACT**tgc**ATATGATCCCC	*Nde*I
W290A	GTTTCTTTGGATCC**gctga**CCAATATGATCC	*Msp*AI
WW-1	TGGGGATCATAT**gcatcagc**GGATCCAAAAGAAACATGGGCG	*Nde*I + *Msp*AI
WW-2	CGCCCATGTTTCTTTTGGATCC**gctgatgc**ATATGATCCCA	*Nde*I + *Msp*AI
W296A	CCAAAAGAAAC**tgca**GCGTTATTAACG	*Pst*I
W301A	GGCGTTATTAAC**agc**G**c**TAGTTTTTGCTATT	*Eco*47III
D291A	TGGAGTTGGG**ca**CCAAAAGAAAC	Δ*Bam*HI
D291E-1	TGGGGATCATATTGGAGTTGGGA**a**CCAAAAGAAACATGGGCG	Δ*Bam*HI
D291E-2	CGCCATGTTTCTTTTGG**t**TCCCAACTCCAATATGATCCCCA	Δ*Bam*HI
D291N-1	TGGGGATCATATTGGAGTTGG**a**ATCCAAAAGAAACATGGGCG	Δ*Bam*HI
D291N-2	CGCCCATGTTTCTTTTGGAT**t**CCAACTCCAATATGATCCCCA	Δ*Bam*HI

WT59$^+$or 2137 cells were bombarded directly onto solid fresh TAP medium containing 150 μg/ml spectinomycin. Plates were incubated under dim light (25 μmol/m^2/s) until colonies appeared. The transformation of gametes was found to be 10–50 times less efficient with respect to the frequency of Spec^R colonies obtained by transformation of vegetative cells.

Purification of Homoplasmic ccsA Site-directed Mutant Strains—Rescued colonies and Spec^R transformants were screened for the presence of the mutant copy of *ccsA* by amplification using *ccsA* specific primers flanking the mutation of interest. Initial Spec^R transformants were found to be predominantly heteroplasmic when vegetative cells were used as recipient cells for transformation but homoplasmic for chloroplast genomes carrying the mutant copy of *ccsA* for the most part when gamete cells were transformed instead. Homoplasmy for *ccsA* mutation could be fixed by inducing gametogenesis of heteroplasmic transformants, followed by one round of subcloning to single colonies on TAP with 700 μg/ml spectinomycin. About 90% of the purified colonies were found to be homoplasmic and remained stable through vegetative growth in the absence of spectinomycin in the medium.

Fluorescence Measurements—The fluorescence imaging system, Fluorcam 700 MF (Photon Systems Instruments Ltd., Brno, Czech Republic), was used to record chlorophyll fluorescence induction and decay kinetics of *Chlamydomonas* cells *in vivo*. Cells were grown on TAP medium under dim light (25 μmol/m^2/s) and dark-adapted for at least 5 min before measurement of the fluorescence emission. Dark-adapted cells were illuminated for 3 to 5 s under actinic light of 60 μmol/m^2/s. Emitted fluorescence was captured by the camera whose sensitivity and shutter were set up at 80% and 1/500 respectively. The collected data were converted into a graph using Excel software (Microsoft).

Protein Preparation and Analysis—For detection of cytochromes, freeze-thaw fractionation and analysis of electrophoretically separated supernatant and pellet fractions by immunodetection or a heme staining procedure have been described previously (47, 52). Enriched thylakoid membrane fractions were prepared from sonicated cell lysates according to Howe *et al.* (47). The 33-kDa protein previously recognized as CcsA (51) was later identified as cyt *f* and all subsequent attempts to generate specific antisera thus far have failed to detect the endogenous CcsA protein (B. W. Dreyfuss and S. S. Nakamoto, unpublished). Polyclonal antisera raised against *C. reinhardtii* cyt c_6, cyt *f* fusion protein, Trx-Ccs1 fusion protein (59), CF_1 and plastocyanin were used for immunodetection. Bound antibodies were detected by alkaline phosphatase-conjugated secondary antibodies.

Enriched thylakoid samples were subjected to Blue Native polyacrylamide gel electrophoresis (68, 69). All steps were carried out at 4 °C. Immediately after preparation, enriched thylakoid membrane samples corresponding to 100 μg of Chl were pelleted by microcentrifugation for 15 min at 4 °C and resuspended in 90 μl of 750 mM ϵ-aminocaproic acid, 50 mM BisTris, pH 7.0. The membranes were then solubilized with 10 μl of dodecylmaltoside (1% final) for 5 min, followed by centrifugation at 20,000 g for 30 min to remove insoluble material. The supernatant was supplemented with 5 μl Coomassie-blue solution (5% Serva Blue G-250/500 mM ϵ-aminocaproic acid) to produce a detergent/Coomassie ratio of 4:1(g/g) and loaded directly onto the gel. In order to estimate the size of the CCS complex, high molecular weight markers (Amersham Biosciences) were prepared in detergent and Coomassie Blue and loaded onto the gel, along with the membrane samples. Blue Native gels consisted of a separating gel (gradient of 6–12% acrylamide) and a stacking gel (4%). Electrophoresis was initiated with cathode buffer containing 0.02% Coomassie-blue at 100 V for ~16 h (1.5 mm x 14 cm), after which the buffer was exchanged with cathode buffer lacking Coomassie-blue and electrophoresis continued for 6 h. Blue-native gels were electrophoretically transferred to 0.2 μm PVDF membranes using the cathode buffer as a transfer buffer at 100 V for 90 min at 4 °C. Membranes were immunodecorated with Ccs1 antisera overnight and bound antibodies detected by alkaline phosphatase-conjugated antibodies using CDP-Star chemiluminescence reagent (Roche Molecular Biochemicals). Immunoblots were exposed to Kodak Bio Max film for 10 min prior to development.

RESULTS

Conservation of CcsA in Plastid and Cyanobacterial Genomes—An alignment of 27 CcsA homologs from 22 plastid genomes from plants and algae and 5 cyanobacterial genomes (see Fig. in supplemental data) shows that CcsA is a well conserved protein (32–43% identity, 43–54% similarity in pairwise alignment with *Chlamydomonas* CcsA) particularly in its C-terminal part. Putative heme-binding residues in CcsA include the WWD signature motif and three invariant histidine residues. Histidinyl residues are of particular interest in the context of heme binding as they are known to be virtually universal proximal and common distal heme ligands in other heme containing proteins such as cytochromes and globins. A key step in understanding the structure and function of CcsA is the determination of the orientation (*p*-side *versus n*-side) of its hydrophobic and hydrophilic domains, in particular the WWD motif and conserved histidines, with respect to the membrane.

Topological Arrangement of CcsA—We decided to deduce the topology of CcsA in a bacterial system using alkaline phosphatase (*phoA*) and the α peptide of β-galactosidase (*lacZα*) as

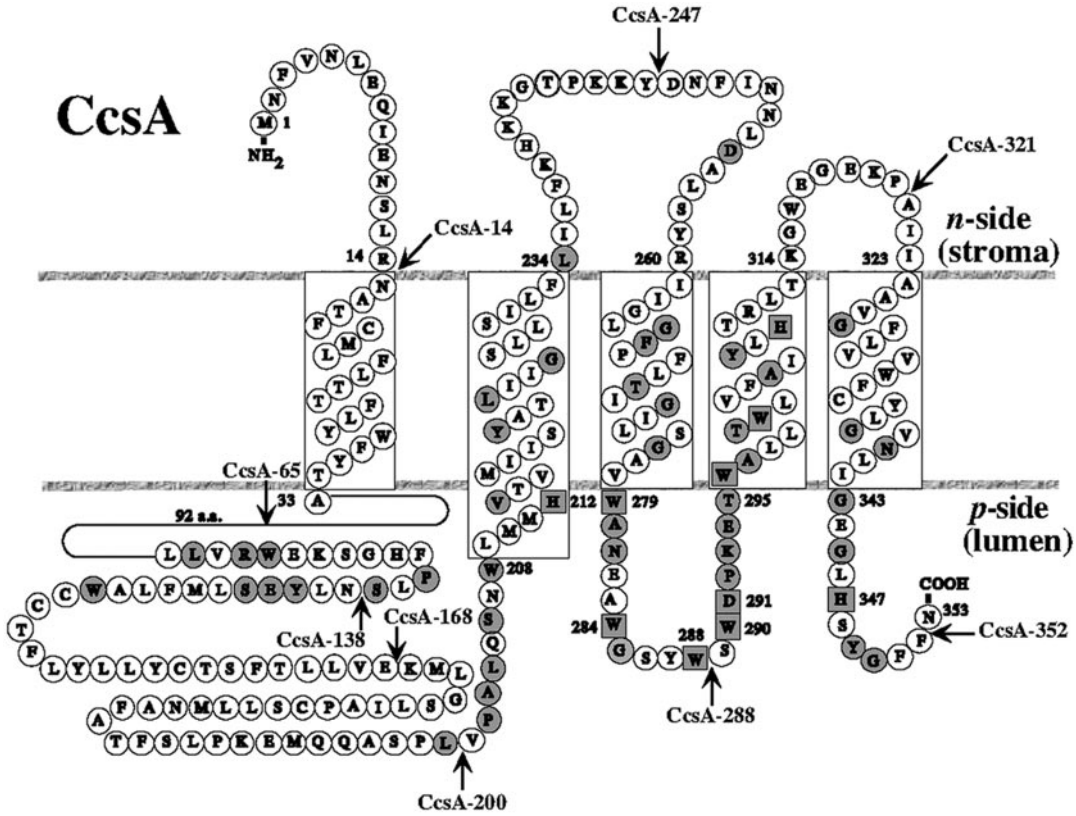

FIG. 1. **Topology of *Chlamydomonas* CcsA.** The topological arrangement of *Chlamydomonas* CcsA was drawn according to PhoA/LacZ fusion analysis in bacteria. The *p*-side (positive side) corresponds to the lumen of the thylakoid and the *n*-side (negative side) corresponds to the stroma of the chloroplast. Large rectangles feature the transmembrane helices of CcsA as predicted from *in silico* analysis using the PHDhtm algorithm (70). The *arrows* indicate, within the CcsA polypeptide, the positions of in frame alkaline phosphatase and β-galactosidase α peptide fusions. Fusions at position 14, 65, 138, 168, 200 247, 288, 321 and 352 are indicated on the drawing by *arrows*. Residues in *gray* are strictly conserved in all 22 plastid and 5 cyanobacterial CcsA homologs (see Fig. in Supplemental Data). Candidate heme binding residues in a square were tested for function by site-directed mutagenesis of the *ccsA* gene in Chlamydomonas.

topological reporters because of their reliability in many experimental systems. The PhoA fusions were engineered in frame to all the predicted soluble loops of the CcsA protein (Fig. 1). A neural network system was utilized to predict the topological arrangement of CcsA (70). Regions which are lumenal in the chloroplast correspond to the *p*-side of the membrane and are expected to be periplasmic. Reciprocally, stromal domains are on the *n*-side of the membrane and are predicted to be exposed in the cytoplasm. Because alkaline phosphatase activity requires oxidative-folding of the protein in the periplasmic space, CcsA-PhoA fusions at periplasmic positions should exhibit high PhoA activity whereas those which are on the cytoplasmic side should exhibit lower activity. Conversely, β-galactosidase being active in the cytoplasm, CcsA-LacZα fusion constructs should show high activity only if the fusion junction is cytoplasmic. As shown in Table II, CcsA fusion products at positions 65, 138, 168, 200, 288 and 352, predicted to be periplasmic, display high alkaline phosphatase activity. All activities are about 8–15-fold above the level of vector control pRGK200 which expresses a cytoplasmically inactive alkaline phosphatase that is devoid of the signal sequence for translocation in the periplasm (18). These results confirm the periplasmic localization of the junctions tested and we can deduce that they have a lumenal localization in the chloroplast.

Plasmids expressing CcsA-LacZα at positions 14, 247 and 321 show a significantly higher level of β−galactosidase activity compared with the empty vector control (see Table II). In accordance with these results, the CcsA-PhoA fusion at position 65, for which we have assigned a periplasmic location, exhibits a very low β−galactosidase activity, about 7–9-fold less than all other CcsA-LacZα fusions. The cytoplasmic location of positions 14, 247, and 321 can be confirmed unambiguously and we are confident in ascribing a stromal location for these positions in the plastid context. Multiple copies of the FLAG or His_6 epitopes can be introduced via an engineered restriction site between residues 243 and 244 without affecting CcsA in Chlamydomonas, which is consistent with the extramembrane stromal orientation of this region of the polypeptide

TABLE II
CcsA-PhoA and -LacZ activities

Alkaline phosphatase and β-galactosidase activities of CcsA fusion proteins expressed in bacteria were measured as described under "Experimental Procedures." At least two representatives of each CcsA fusion that was generated by PCR were tested for activity. The value is indicated as the mean and S.D. of three independent measurements for the two representatives of each fusion. *n*-Side and *p*-Side correspond to the negative and positive sides of the membrane, respectively. ND, not determined.

	PhoA activity	*LacZ* activity	Topology
	Miller units	*units*	
pRGK200	17 (±3)	0	
pccsA-14	18 (±3)	43 (±6)	*n*-Side
pccsA-65	130 (±12)	6 (±2)	*p*-Side
pccsA-138	237 (±31)	ND	*p*-Side
pccsA-168	139 (±12)	ND	*p*-Side
pccsA-200	246 (±30)	ND	*p*-Side
pccsA-247	13 (±2)	42 (±6)	*n*-Side
pccsA-288	145 (±17)	ND	*p*-Side
pccsA-321	16 (±3)	56 (±10)	*n*-Side
pccsA-352	199 (±15)	ND	*p*-Side

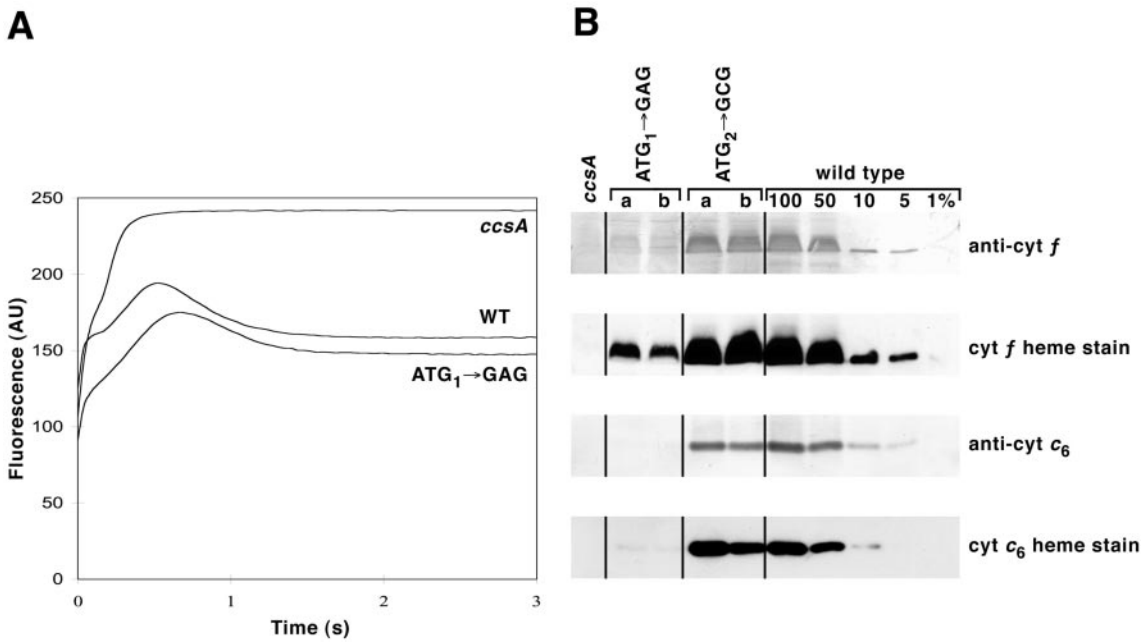

FIG. 2. **Phenotypic analysis of $ATG_1 \rightarrow GAG$ and $ATG_2 \rightarrow GCG$ ccsA mutants.** *A*, fluorescence kinetics of $ATG_1 \rightarrow GAG$ *ccsA* mutant. The fluorescence induction and decay kinetics observed in a dark to light transition for the $ATG_1 \rightarrow GAG$ *ccsA* mutant is shown in comparison with those from the wild type (WT) and B6 (*ccsA*) strains. Only one representative transformant is presented here. The continuously rising curve for the *ccsA* mutant is typical of a specific block in electron transfer at the level of cytochrome b_6f complex due to its impaired assembly in the absence of membrane-bound holocytochrome *f*. The strains were grown on TAP agar for 3 days before recording the fluorescence in arbitrary units (*AU*) over a 3 s illumination period. *B*, accumulation of plastid c-type cytochromes in $ATG_1 \rightarrow GAG$ and $ATG_2 \rightarrow GCG$ *ccsA* mutants. Protein fractions were prepared from copper-deficient cultures of wild type (2137), *ccsA* (B6) and 2 representatives (*a* and *b*) of each $ATG_1 \rightarrow GAG$ and $ATG_2 \rightarrow GCG$ *ccsA* strains generated by chloroplast transformation. The supernatant fractions were analyzed for holocyt c_6 accumulation (anti-cyt c_6 and heme stain) whereas the pellet fraction was used to assess holocyt *f* abundance (anti-cyt *f* and heme stain). All the samples were found to be copper-deficient based on the absence or very low abundance of immunoreactive holoplastocyanin (data not shown). Hence, cyt c_6 should be expressed in all samples. Samples corresponding to 5 μg of Chl were separated in SDS-containing acrylamide (12%) gels or native acrylamide gels (15%) to detect cyt *f* and cyt c_6 respectively. For an estimation of the cytochrome abundance, dilutions of the wild type sample were loaded on the gel. Equal loading of the samples was tested by Coomassie Blue staining (not shown). Gels were then transferred to PVDF membranes following electrophoresis before heme staining by chemiluminescence and immunodecoration with antisera against *Chlamydomonas* cyt *f* and cyt c_6 (79). Heme staining and autoradiographic exposures were performed simultaneously for all samples.

(data not shown). Overall, the data establish the polytopic arrangement of plastid CcsA in the membrane with five transmembrane helices and N and C termini facing the stroma and lumen, respectively (Fig. 1). Based on this topology, the WWD motif and the conserved histidinyl residues His^{212} and His^{347} are located on the lumenal side. The conserved residue His^{309} appears to be located on the stromal side, perhaps within a transmembrane segment. We then addressed the question of the importance of the conserved residues for *Chlamydomonas* CcsA function and structure by site-directed mutagenesis of *ccsA*.

Functional Analysis of CcsA in Plastid—A preliminary assessment of the effect of site-directed mutations in CcsA can be obtained rapidly by testing all mutated alleles for their ability to rescue the photosynthetic deficiency of a *ccsA* mutant. Restoration of photosynthesis relies only on the restoration of holocyt *f* accumulation. Mutations that have no or only a marginal effect on function should rescue the photosynthetic deficiency at frequencies comparable with wild type whereas those that do affect function should not rescue. In order to obtain a precise and quantitative evaluation of the function of CcsA, rescued colonies are then assayed for the unselected phenotype, *i.e.* the accumulation of holocyt c_6 in copper-deficient conditions. Mutations that do not rescue are analyzed by replacing, through homologous recombination in the plastid, the wild type *ccsA* gene with the mutated version of *ccsA*. This was accomplished either via constructs which have *ccsA* physically linked to the *aadA* gene or by co-transforming the mutated *ccsA* together with the $Spec^R$16 S rRNA gene. Both *aadA* and $Spec^R$ 16 S rRNA genes confer resistance to spectinomycin.

Determination of the Initiation Codon—Analysis of the longest ORF in *Chlamydomonas ccsA* indicates the presence of two in-frame ATGs (ATG_1 and ATG_2), 19 codons apart (51). Because neither ATG is associated with a typical consensus Shine-Dalgarno sequence, it was not clear which codon functions as the initiation site for the translation of the CcsA polypeptide. To address this question, both ATG_1 and ATG_2 codons were mutated to GAG (aspartic acid) and GCG (alanine) codons and the resulting sequences tested for their ability to rescue the photosynthetic deficiency of *ccsA*-B6 strain. Neither of these latter codons are known to function as initiation codons in the plastid context. The *ccsA*-B6 strain was chosen as a recipient because it carries a single nucleotide deletion causing a premature termination of translation at codon 23 of the *ccsA* gene (49). We reasoned that the proximity of this mutation to the introduced mutations at positions 1 and 20 should preclude a high frequency of recombination events that could result in reconstruction of a wild type *ccsA* gene. The frequency of photosynthetically proficient transformants recovered after transformation of *ccsA*-B6 strain is comparable when transforming with a wild type *ccsA* gene (116 ± 3 transformants, $n = 3$ transformations) or with the *ccsA* allele carrying the $ATG_2 \rightarrow GCG$ mutation (153 ± 23 transformants, $n = 3$ transformations) whereas no transformants were recovered with the $ATG_1 \rightarrow GAG$ *ccsA* allele (3 independent transformations) or the no DNA control. Homoplasmic $ATG_2 \rightarrow GCG$ transformants were indistinguishable from wild type transformants in that they displayed the same fluorescence induction and decay kinetics and accumulated wild type levels of holoforms of both cyt *f* and cyt c_6 (data not

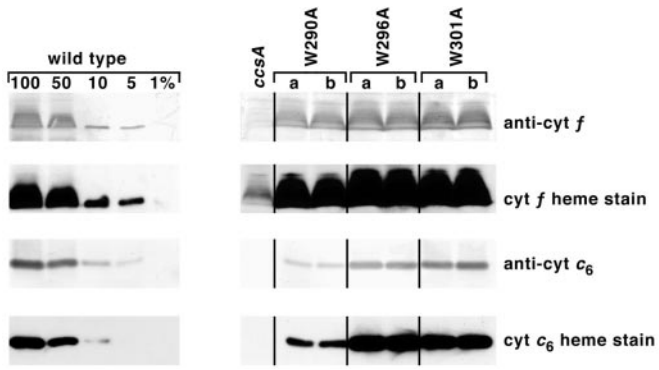

FIG. 3. **Accumulation of plastid c-type cytochromes in W290A, W296A, and W301 mutants.** Protein fractions were prepared from copper-deficient cultures of wild type (2137), *ccsA* (ct59) strains and 2 representatives (*a* and *b*) of W290A, W296A and W301 transformants generated by biolistic transformation. The supernatant fractions were analyzed for holocyt c_6 accumulation (anti-cyt c_6 and heme stain) whereas the pellet fraction was used to assess holocyt *f* abundance (anti-cyt *f* and heme stain). All the samples were found to be copper-deficient based on the absence or very low abundance of immunoreactive holoplastocyanin (data not shown). Hence, cyt c_6 should be expressed in all samples. Samples corresponding to 5 μg Chl were separated in SDS-containing acrylamide (12%) gels or native acrylamide gels (15%) to detect cyt *f* and cyt c_6 respectively. Equal loading of the sample was tested by Coomassie blue staining (not shown). Gels were then transferred to PVDF membranes following electrophoresis before heme staining by chemiluminescence and immunodecoration with antisera against *Chlamydomonas* cyt *f* and cyt c_6. The dilution series of wild type for heme stain and immunodecoration were developed in parallel with the experimental samples from the mutant strains.

shown and Fig. 2, *A* and *B*). The $ATG_2 \rightarrow GCG$ mutation fully rescues the function of a *ccsA* mutant whereas the $ATG_1 \rightarrow GAG$ cannot, suggesting that the first ATG is critical for the function of the CcsA polypeptide.

We therefore generated $Spec^R$ $ATG_1 \rightarrow GAG$ mutants under non-selective conditions (*i.e.* heterotrophic growth). Fluorescence transients of homoplasmic $Spec^R$ $ATG_1 \rightarrow GAG$ transformants appear wild type, albeit with slightly slower decay kinetics (Fig. 2*A*) and the transformants were able to grow photosynthetically under light intensities from 50 to 170 μmol/m^2/s, but very poorly at 500 μmol/m^2/s, suggesting that photosynthesis is affected but not completely compromised (data not shown). Indeed, we showed that, although significantly reduced, some holocyt *f* and c_6 accumulation occurs in the plastid of homoplasmic $ATG_1 \rightarrow GAG$ mutants (Fig. 2*B*). The reduced accumulation of holoforms of cyt *f* (<20%) and cyt c_6 (<5%) in these transformants suggests that some translation of the *ccsA* gene is rendered possible, presumably at ATG_2 or alternatively at the GAG codon as there is no other in-frame ATG or non orthodox initiator codon upstream of ATG_1. The first possibility is more likely since the GAG codon has not been shown previously to function as an initiator codon in any system investigated so far. Because ATG_2 can function as an initiator when ATG_1 is mutated, we infer that the first 20 amino-acids of CcsA are not absolutely essential for targeting of the polypeptide to the thylakoid membrane, but that the first ATG serves normally as the preferred initiation codon for the translation *in vivo* of the CcsA polypeptide.

Mutagenesis of Candidate Heme-binding Residues—The tryptophan residues Trp^{284}, Trp^{288}, and Trp^{290} lying within the WWD motif of CcsA and the neighboring residues Trp^{279}, Trp^{296}, and Trp^{301} (Figs. 1 and 3*A*) were tested for function by directed mutagenesis to alanine residues. The non-photosynthetic strain ct59 carrying a frameshift mutation at position 269 of the *ccsA* gene has a low frequency of reversion to photosynthetic proficiency ($<10^{-9}$) and therefore was selected as a recipient strain for complementation experiments. We reasoned that the recovery of photosynthetic transformants that arise from a recombination event between residues 269 and 279–301 should be a rare event. Thus, all photosynthetic transformants recovered should harbor the introduced mutation. Plasmids pW279A, pW284A, pW288A, pW290A and pW296A were introduced into *ccsA*-ct59 by chloroplast transformation. Only pW290A, pW296A and pW301A mutations were able to complement the photosynthetic deficiency of strain *ccsA*-ct59 suggesting that Trp^{279}, Trp^{284}, and Trp^{288} are critical for the function of CcsA.

The abundance of holoforms of cyt c_6 and cyt *f* was not affected in homoplasmic W296A and W301A transformants. However, holocyt *f* accumulation was reduced to 50% and holocyt c_6 accumulation was reduced to 20% of the corresponding wild type level in the W290A mutant (Fig. 3*B*). Although the W290A mutant is able to grow photosynthetically, its ability to assemble *c*-type holocytochromes is diminished. We conclude that Trp^{290} is required for full function of CcsA whereas Trp^{296} and Trp^{301} are not. Homoplasmic W279A-*aadA* and W284A-*aadA* transformants failed to grow on minimal medium under all light intensities (40–170-500 μmol/m^2/s) indicating that holocyt *f* assembly is severely impaired in those mutants. The fluorescence induction and decay kinetics are identical to those of a *ccsA* mutant and characteristic of b_6f deficient mutants due to compromised assembly of the b_6f complex in the absence of membrane-anchored holocyt *f* (data not shown). Immunoblot and heme stain analysis showed that the W279A mutation blocks completely the assembly of both holocyts *f* and holocyt c_6 (Fig. 4). In the W284A-*aadA* transformants, a small amount of apocyt *f* could still be detected immunochemically (Fig. 4). In the course of our study, we observed that authentic apocyt *f* can accumulate to various levels in strains carrying a *ccsA*-null allele and also in other *ccs* mutants. The amount of apocyt *f* which accumulates does not appear to correlate to the molecular lesion but may be dependent upon genetic background and cell density.

The W288A-*aadA* mutant was able to grow photosynthetically at all light intensities, albeit to a lower extent compared with the W290A-*aadA* mutant and the wild type-*aadA* control strain. (Fig. 5*A*). Note that based on the immunoblot and heme stain analysis, it is evident that the level of holocytochrome *c* maturation is much less in the W290A-*aadA* strains (Fig. 4) compared with the W290A transformants obtained by rescue of the *ccsA*-B6 strain (Fig. 3). The genetic background of the two original strains (*ccsA*-B6 and $WT59^+$) used to generate the transformants might account for the difference we observed.

The fact that Trp^{288} and Trp^{290} lie close to one another within the conserved WWD motif and that neither alanine substitution completely abolished CcsA function led us to assess the phenotype of the double mutation W288AW290A. The W288AW290A-*aadA* strain was unable to sustain photosynthetic growth under 500 μmol/m^2/s illumination although it displayed some residual growth at 50 and 170 μmol/m^2/s (Fig. 5*A* and data not shown). The level of photosynthetic growth on minimal medium of the W288A-*aadA* and W288AW290A-*aadA* mutants correlated with the level of holocyt *f* accumulated as shown by immunoblot and heme stain analysis (Fig. 4). Holocyt c_6 conversion seems to be affected to the same extent as holocyt *f* by these mutations (Fig. 4). The W288AW290A cells displayed typical *ccsA*-null mutant fluorescence transient (Figs. 4, 5*B*) but the low residual level of holocyt *f* (2%) appears to be enough to sustain photosynthetic growth up to a light intensity of 170 μmol/m^2/s.

The aspartate residue Asp^{291} was also tested for functional importance by mutating it to glutamate, asparagine or alanine

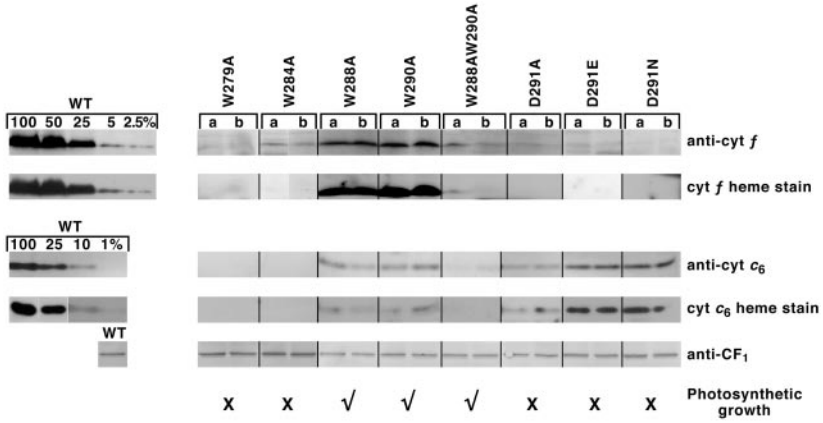

FIG. 4. **Accumulation of plastid c-type cytochromes in the tryptophan, aspartic acid, and histidine ccsA mutants.** Protein fractions were prepared from copper-deficient cultures of wild type-*aadA* (WT) and 2 representatives (*a* and *b*) of W279A-*aadA*, W290A-*aadA*, W288AW290A-*aadA*, D291A-*aadA*, D291E-*aadA* and D291N-*aadA* transformants generated by chloroplast transformation. The supernatant fractions were analyzed for holocyt c_6 accumulation (anti-cyt c_6 and heme stain) whereas the pellet fraction was used to assess holocyt *f* abundance (anti-cyt *f* and heme stain) and CF_1 of the ATPase (anti CF_1) as a loading control. All the samples were found to be copper-deficient based on the absence or very low abundance of immunoreactive holoplastocyanin (data not shown). Hence, cyt c_6 should be expressed in all samples. Samples corresponding to 15 μg Chl were separated in SDS-containing acrylamide (12%) gels or native acrylamide gels (15%) to detect cyt *f* and cyt c_6 respectively. Samples corresponding to 3 μg Chl were separated in denaturing acrylamide gel to detect CF_1. Following electrophoresis, gels were then transferred to PVDF membranes before heme staining by chemiluminescence and immunodecoration with antisera against *Chlamydomonas* cyt *f*, cyt c_6 or CF_1. For an estimation of the cytochrome abundance, dilutions of the wild type sample were loaded on the gel. Heme staining exposure representing equivalent intensity for wild type controls in all experiments are shown. The ability and inability of the transformants to grow photosynthetically is indicated by "✓" and "X," respectively.

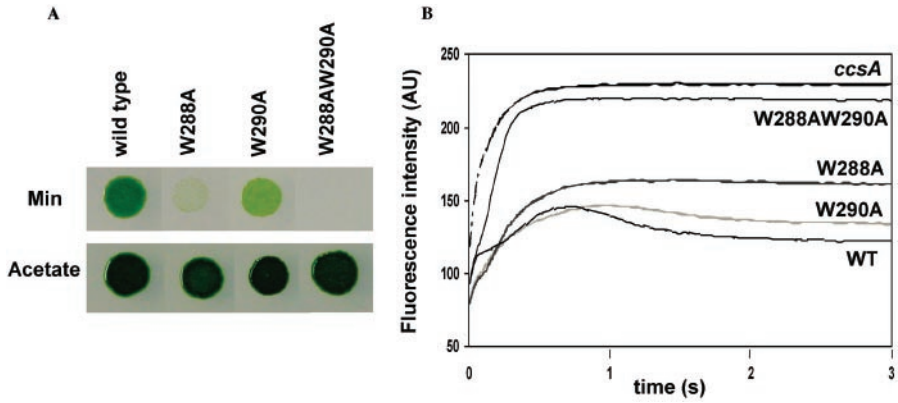

FIG. 5. **Tryptophan 288 and 290 of the WWD motif are required for photosynthesis.** *A*, photosynthetic growth of W288A, W290A and W288AW290A mutants. Equal numbers of cells were grown photosynthetically (Min) under high light (500 μmol/m^2/s) or heterotrophically (Acetate) under dim light (25 μmol/m^2/s) for 10 days at 25 °C. *B*, fluorescence induction and decay kinetics of W288A, W290A and W288AW290A mutants. Fluorescence transients of W288A-*aadA*, W290A-*aadA*, W288AW290A-*aadA*, *ccsA* and wild type-*aadA* strains were measured on colonies grown for 4 days on solid TAP medium using Fluorcam 700 MF from Photon Systems Instruments. The strains were plated on TAP agar 3 days before recording the fluorescence in arbitrary units (AU) over a 3 s illumination period.

residue. None of the mutations rescued the photosynthetic growth of *ccsA*-ct59 arguing for the absolute requirement of the aspartic residue in the assembly of holocyt *f*. Indeed, the D291E-*aadA*, D291N-*aadA* and D291A-*aadA* strains are similar to a *ccsA*-null mutant. They were unable to grow photosynthetically, displayed b_6f mutant fluorescence induction and decay kinetics and did not accumulate holocyt *f* (data not shown and Fig. 4). Surprisingly, when analyzed for the unselected phenotype (*i.e.* holocyt c_6 assembly), the D291A, D291E, and D291N mutations still enabled the assembly of appreciable amounts of holocyt c_6 (Fig. 4). It seems that the D291E and D291N mutations have less effect on holocyt c_6 maturation than does the D291A substitution.

To assess the importance of the histidinyl residues, the mutants H212D, H309E, H347E, H347A were constructed. All mutations failed to restore photosynthesis to a *ccsA* mutant and all spectinomycin-resistant transformants carrying either mutation were fully deficient for photosynthesis. As shown by immunoblot and heme staining analysis, no accumulation of plastid *c*-type cytochromes was detectable demonstrating that all three histidinyl residues are critical for the activity of CcsA (Fig. 6). We conclude that all three conserved histidine residues as well as the tryptophan-rich region of CcsA are functionally important for the assembly of plastid *c*-type holocytochromes.

A CCS Complex within the Thylakoid Membranes—Based on the clustering of *ccsA*-like and *CCS1*-like genes in bacteria, Ccs1 is likely to be a candidate interacting partner of CcsA. To look at the presence of a CCS complex in the thylakoid membrane, we chose the technique of BN-PAGE (71, 72). Thylakoid membrane fractions from the wild type strain, *ccs1* and *ccsA* mutants, and a transformant restored to photosynthetic competence by complementation with the wild type *ccsA* gene were analyzed by this technique. Several discrete complexes including green bands representing Chl-containing complexes can be observed (Fig. 7). The intensely green band at ~100 kDa was confirmed to be the trimeric form of the major light-harvesting complex of the photosystem II by subsequent fully denaturing electrophoresis in the second dimension (data not shown). The dimeric form of the b_6f complex (250 kDa) could be detected by

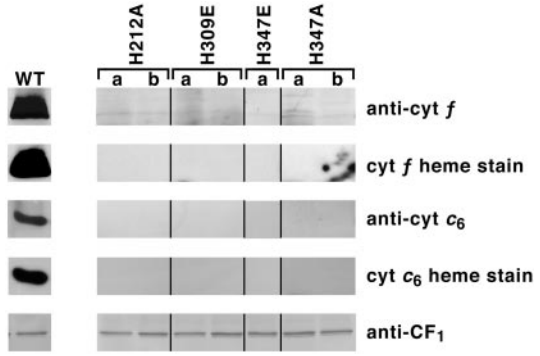

FIG. 6. **Invariant histidine residues are essential for the assembly of plastid *c*-type cytochromes.** Protein fractions were prepared from copper-deficient cultures of wild type-*aadA* (WT), 2 representatives (*a* and *b*) of H213A-*aadA*, H309A-*aadA*, H347A-*aadA*, and one representative (*a*) of H349E-*aadA* transformants generated by chloroplast transformation. The supernatant fractions were analyzed for holocyt c_6 accumulation (anti-cyt c_6 and heme stain) whereas the pellet fraction was used to assess holocyt *f* abundance (anti-cyt *f* and heme stain) and CF_1 of the ATPase (anti-CF_1) as a loading control. All the samples were found to be copper-deficient based on the absence or very low abundance of immunoreactive holoplastocyanin (data not shown). Hence, cyt c_6 should be expressed in all samples. Samples corresponding to 15 μg Chl were separated in SDS-containing acrylamide (12%) gels or native acrylamide gels (15%) to detect cyt *f* and cyt c_6 respectively. For an estimation of the cytochrome abundance, dilutions of the wild type sample were loaded on the gel. Samples corresponding to 3 μg Chl were separated in denaturing acrylamide gel to detect CF_1. Following electrophoresis, gels were then transferred to PVDF membranes before heme staining by chemiluminescence and immunodecoration with antisera against *Chlamydomonas* cyt *f*, cyt c_6 or CF_1. Heme staining exposure representing equivalent intensity for wild type controls in all experiments are shown.

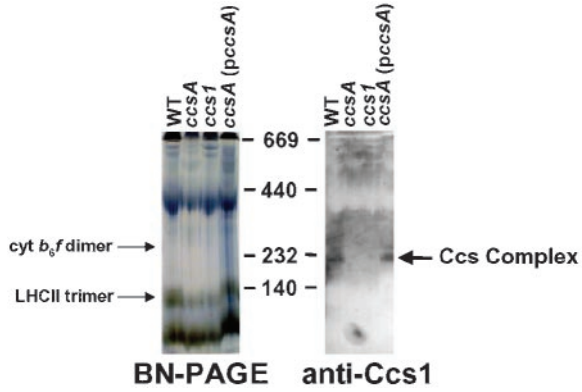

FIG. 7. **A "CCS" complex in the thylakoid membranes.** Thylakoid membrane enriched fractions from wild type CC125 (WT), *ccsA*-B6 (*ccsA*), *ccs1–7*::*ARG7* (*ccs1*) strains and a phototrophic transformant generated by complementation of *ccsA*-B6 strain with a plasmid carrying the *ccsA* gene (p*ccsA*) were solubilized with dodecylmaltoside. Samples corresponding to 100 μg of Chl were subjected to BN-PAGE (*left side*) and transferred to 0.2 μm PVDF membranes before immunodecoration with an anti-Ccs1p antibody (*right side*). The positions of the dimeric form of the cytochrome b_6f complex (cyt b_6f) and the trimeric form of the light-harvesting complex of photosystem II (LHCII) are indicated by an *arrow* on the BN gel. The position of the 200-kDa complex referred as "CCS" complex and containing Ccs1 is indicated by an arrow on the anti-Ccs1 immunoblot.

immunoblotting with cyt *f* specific antibodies in the wild type and *ccsA*-complemented *ccsA* mutant (data not shown). The migration of both these complexes is consistent with their known masses and previously observed migrations of dodecyl maltoside solubilized spinach thylakoid separated on BN-PAGE (71), indicating proper solubilization and electrophoretic separation of the *Chlamydomonas* thylakoids. The presence of a CCS complex was analyzed by immunoblotting with a Ccs1-specific antibody. An immunoreactive species was observed in thylakoid membranes from the wild type strain but was absent in membranes from either the *ccs1* or *ccsA* strains. This immunoreactive species had a molecular weight of ~200 kDa, suggesting that Ccs1 occurs in the thylakoid membrane as a component of a high molecular weight complex. The observation that Ccs1 is absent in a *ccsA* mutant strain but present in the same strain complemented with the *ccsA* gene suggests that CcsA is required for Ccs1 accumulation and that CcsA is also a component of the 200-kDa complex.

DISCUSSION

In an attempt to extend our current knowledge on the mechanisms of cytochromes *c* assembly in system II, we explored here the function of plastid CcsA as a prototypical protein. In this paper, we provide evidence that: i) CcsA is a polytopic protein with the WWD motif and two conserved histidine residues lying in the lumen and one histidine residue exposed on the stromal side, (ii) the WWD motif and histidinyl residues are important for the function of CcsA in assembling cyt *f* and cyt c_6 in plastid, (iii) a probable CCS complex containing CcsA and Ccs1 is present in thylakoid membranes.

The WWD Motif Is Functionally Important—A recurring theme for this family of proteins is that the WWD motif acts like a hydrophobic platform involved in the binding and presentation of heme. This view is further reinforced by the presence of at least two conserved histidinyl residues, believed to coordinate the heme and lying in loops which bracket the WWD motif. Functional studies addressing the question of the functional importance of the key residues of these WWD proteins in their topological context largely support their role in a transmembrane heme delivery route. It is noteworthy that all experimentally confirmed topology studies have placed the WWD motif and two conserved histidines on the *p*-side of the membrane where the heme attachment reaction was demonstrated to occur (18, 31, 42). By using the well established PhoA and LacZ topological markers in bacteria, we were able to construct a topological model of plastid CcsA. As far as the histidines and tryptophan signature motif locations are concerned, our model is in concordance with other candidate topologies such as that deduced for *Mycobacteria leprae* CcsA (31). The mycobacterial model supports the existence of one additional N-terminal transmembrane domain, compared with its plastid counterpart. This model results in an extra-membrane loop lying on the *n*-side of the membrane which is absent in *Chlamydomonas* CcsA. However, we believe that this topological distinction in the mycobacterial CcsA is probably not functionally meaningful as the region corresponding to the additional transmembrane segment and loop is not conserved in the plastid and cyanobacterial homologs. This topological distinction could reflect the different modes of membrane protein import in bacteria *versus* cyanobacteria/plastids and in that view, one possible function for the additional transmembrane segment in mycobacterial CcsA could be to serve as an uncleaved signal-sequence anchor.

Previous attempts to identify essential residues in polytopic WWD proteins through site-directed mutagenesis have not led to a clear view of how important is the tryptophan-rich motif for the assembly of *c*-type holocytochromes (31, 42, 44–46). This could be due to the fact that the effect of the mutations appears different when examined only at the level of growth (31) or when analyzed for the accumulation of a subset of endogenous *c*-type cytochromes and/or a heterologous cytochrome expressed as reporter of the maturation process (45, 46). In order to give a consistent view of the functional importance of the tryptophan rich domain, we have correlated systematically in our study each site-directed mutation in the *ccsA* gene with the level of both holocyts c_6 and *f* formed in the

plastid and also with its ability to sustain photosynthetic growth. Our experimental data show that any substitution of the tryptophan residues results in a similar effect on the formation of both holoforms of cyt *f* and cyt c_6. Among the six conserved tryptophan residues we tested (Trp^{279}, Trp^{284}, Trp^{288}, Trp^{290}, Trp^{296}, and Trp^{301}), only Trp^{279} and Trp^{284} are strictly essential for *c*-type cytochrome biogenesis. Although there is the possibility that tryptophan residues have a topology-determining role, we favor the idea that they are critical for CcsA activity and/or structure. Indeed, recent studies have established that tryptophan residues in membrane proteins are not topological determinants but could rather fulfill a role as interfacially anchoring residues when located on the trans-side of the membrane (73). The finding that neither simple or double alanine substitutions of the Trp^{288} and Trp^{290} residues in the Trp^{288}-Xaa-Trp^{290}-Asp sequence disrupt completely the assembly process of both holocyt *f* and holocyt c_6 substantiates the concept that the signature motif provides multiple hydrophobic interfaces, possibly for interaction with heme (3). This is also illustrated by the fact that only a residue with an aromatic side chain, such as phenylalanine, can functionally substitute for a tryptophan in a position equivalent to Trp^{290} in *Pseudomonas fluorescens* CcmC whereas any other substitution at this position results in a complete loss of *c*-type cytochrome assembly (42). This is in contrast to other mutagenesis studies of CcmC and CcmF in *E. coli*, where only multiple substitutions of the tryptophan residues within the WWD motif lead to a complete loss of cytochrome *c* maturation (45, 46).

The aspartic residue Asp^{291} in the signature motif is absolutely required for the biogenesis of holocyt *f* as glutamic, asparagine and alanine substitutions completely abolished its assembly. Unexpectedly, we found that any of these changes do not affect to the same extent the formation of holocyt c_6. One likely explanation would be that in addition to its role as a heme presentation surface, the WWD motif also provides a site of interaction with apocytochromes as already suggested by Page *et al.* (24). At present, the question of why this particular acidic residue is needed in this position in the tryptophan motif of CcsA, whereas CcmC function seem to tolerate a glutamic residue in *E. coli* and *P. fluorescens* has no obvious answer (31, 42).

The Histidine Residues Are Critical for CcsA Function—That all histidines residues, when altered, completely disrupt holocytochromes maturation and that we could not obtain by-pass suppressors ($<10^{-9}$, unpublished) underscore their pivotal role in the assembly process. Strictly conserved histidinyl residues located on the *p*-side of the membrane in CcmC and CcmF were also demonstrated to be critical for the activity of the proteins in cytochrome *c* maturation (31, 45, 46) and, similar to our study, no compensatory mutation of the *E. coli* CcmC histidinyl mutants could be isolated (31).

The theme of invariant and essential histidines as heme ligands along with the idea that tryptophans afford several sites of contact for heme handling makes in our sense the heme delivery function of CcsA the most compelling hypothesis. Recently, the implication of the WWD motif and conserved histidines in heme-binding has been questioned by the observation that a CcmC mutant with mutated histidines and all tryptophan and aspartic residues of the motif changed to alanines is fully deficient for cytochrome *c* maturation but remains unaltered in its binding capacity to heme in an hemin-agarose assay (45). Based on the fact that such a mutant is defective for its interaction with CcmE, the WWD motif and histidines are believed to be required for a functional interaction with the heme chaperone CcmE. However, the closer evolutionary relationship between CcmC and CcsA (5), suggestive of an equivalent function of these two proteins in cytochrome maturation, and the absence of a CcmE-like heme chaperone in system II bacterial genomes makes in our view the role of the WWD motif as a CcmE interaction interface less likely. On the other hand, the crystal structure of the heme-hemopexin (Hpx) complex revealed that the heme ligand is bound to two histidines residues in a pocket formed by aromatic residues including tryptophans (74). This supports the idea that in the WWD cytochrome *c* biogenesis proteins, tryptophan and histidine could also provide a transient binding site for the heme during the assembly of cytochromes *c*.

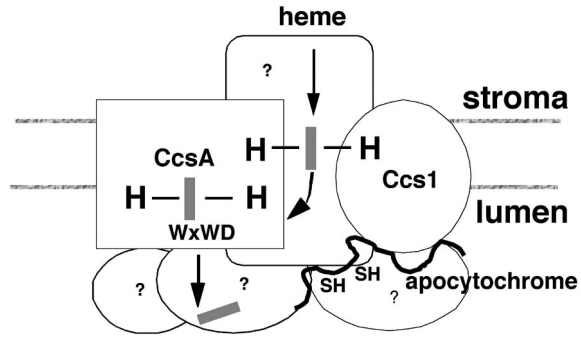

FIG. 8. **Model for a c-type cytochromes assembly machinery in plastid.** Our current working model of a *c*-type cytochromes assembly apparatus in the thylakoid membrane includes plastid-encoded CcsA, nucleus-encoded Ccs1 and other unidentified components (?). Essential conserved histidinyl residues (H) in CcsA and Ccs1 (see accompanying paper) are positioned in the lumen and within the membrane close to the stromal side. Heme is represented as a *gray rectangle* and we hypothesize it is handled from the stroma to the lumen side through histidinyl residues and the WWD motif, for delivery and ligation to reduced apocytochromes (SH indicate the sulfhydryl groups of the C*XX*CH motif) "chaperoned" by Ccs1.

CcsA-Ccs1, A Functional Subcomplex Involved in Heme Delivery—How does CcsA operate and function in the pathway? Evidence for a high molecular weight complex containing Ccs1 within the thylakoid membrane that is no longer detected in the absence of CcsA suggests that Ccs1 could be a putative partner of CcsA (Fig. 8). An attractive hypothesis is that the invariant and essential histidines predicted to be exposed to the stroma in both CcsA, a unique feature of this WWD family representative, and Ccs1 (59) provide an entry site for heme on the stromal side. However, our topology and mutagenesis studies do not elucidate whether these residues are in the membrane or exposed to the stroma and how heme is captured from the *n*-side of the membrane to be delivered to the site of cytochrome *c* assembly is not known in any of the three systems (6, 28). Our current working model postulates that heme is relayed through the histidine residues from stroma to lumen and is presented on the WWD platform to the apocytochromes chaperoned by Ccs1 (Fig. 8). Whether CcsA is also involved in the ligation reaction of heme to the apocytochromes still remains undetermined. Prevalent models for a heme delivery pathway to apocytochromes in system I favor CcmF as the presumptive heme lyase *versus* CcmC which is required earlier in cytochrome *c* maturation (28, 46). In *Wolinella succinogenes*, an ϵ-protebacterium with a possible system II for cytochrome *c* maturation, the NrfI protein is proposed to result from a fusion of a protein predicted to be topologically similar to Ccs1 and a CcsA-like moiety (75). The fact that NrfI is only dedicated to the attachment of heme to the unusual CXXCK motif in pentaheme *c*-type cytochrome NrfA argues in favor of a heme handling/ligation activity of CcsA and reinforces the view of a CcsA-Ccs1 functional subcomplex (76). It remains to be determined if NrfI is only

involved in heme delivery to NrfA or functions also in the transport of heme from the *n*-side of the membrane for ligation to the CXXCK motif.

Other components of the high molecular weight complex detected in the thylakoid membrane are likely to correspond to the gene products of the unidentified *CCS2* to *4* loci (59). We believe that this complex acts as a site for *c*-type cytochromes maturation and is composed of other factors whose biochemical activities are required to complete holocytochrome assembly. Such candidate factors might include components required to maintain apocytochrome cysteinyl thiols and heme in a reduced state, compatible with the lyase reaction. Evidence for a membrane-bound thiol-reducing subpathway for apocytochromes *c* in the "CCS" pathway was provided recently in bacterial models with the discovery of CcdA/DipZ, a protein first identified in system I and involved in conveying reducing equivalents across the membrane, and CcsX a putative periplasmic thioredoxin (57, 58, 77, 78). The existence of a redox subpathway in the plastid system is suspected based on the presence of a *CcdA*-like gene in the rhodoplast genome of *P. purpurea* and in the nuclear genome of both[3] *Chlamydomonas* and *Arabidopsis thaliana* (6). In Chlamydomonas, preliminary results showing that some *ccs* mutants are restored for photosynthetic growth upon addition of exogenous reducing thiol compounds in the medium, whereas *ccsA* and *ccs1* mutants are not, indicate that this thiol-reducing pathway in cytochrome *c* biogenesis also functions in plastid.[4]

Finally, we established also that the transformation of *Chlamydomonas* gametes significantly improves the generation of homoplasmic chloroplastic transformants. Even though we did not carry out a comparative study for all the site-directed mutants we generated, the ease with which homoplasmic transformants are obtained when gametes are used as recipient cells suggests that this approach could be a useful one. Gametes undergo a reduction of the copy number of their chloroplast genomes (60) and this probably facilitates the segregation of the molecules carrying the introduced DNA. Our method should be preferred over the 5-fluorodesoxyuridine treatment of vegetative host cells prior to transformation which also reduces the copy number of the plastid genomes but has a mutagenic effect on the plastome.

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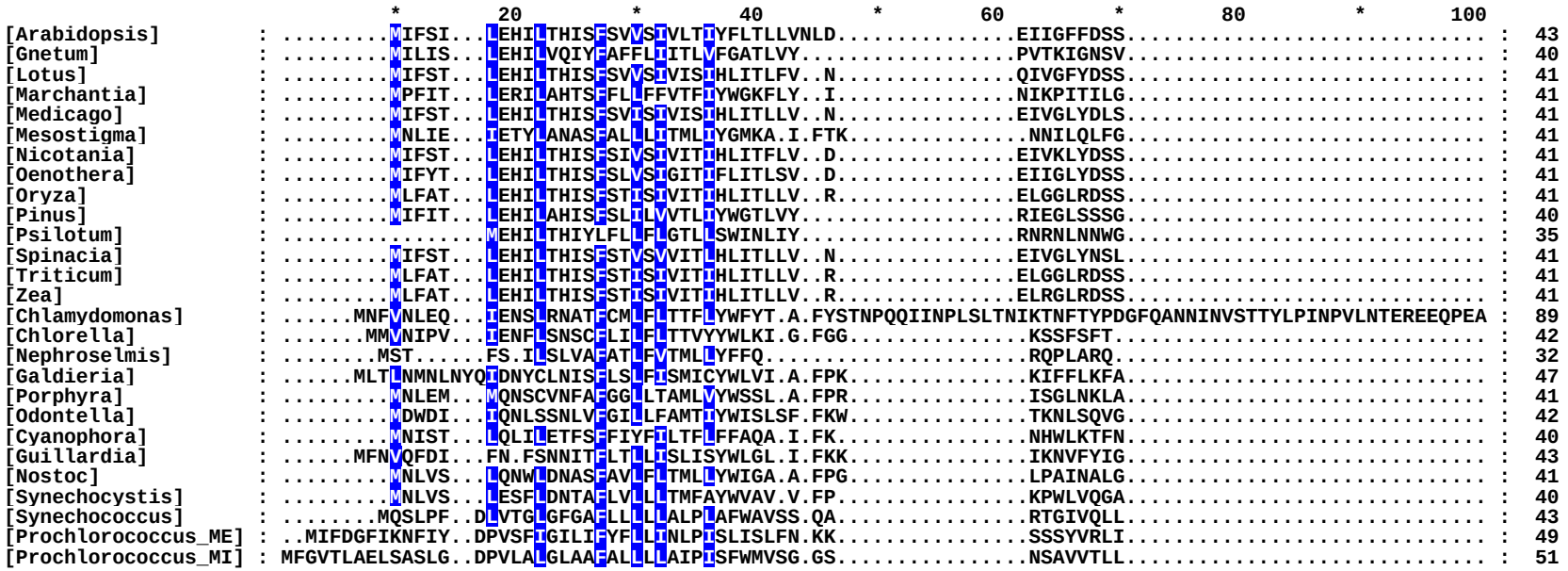
Figure Supplemental data



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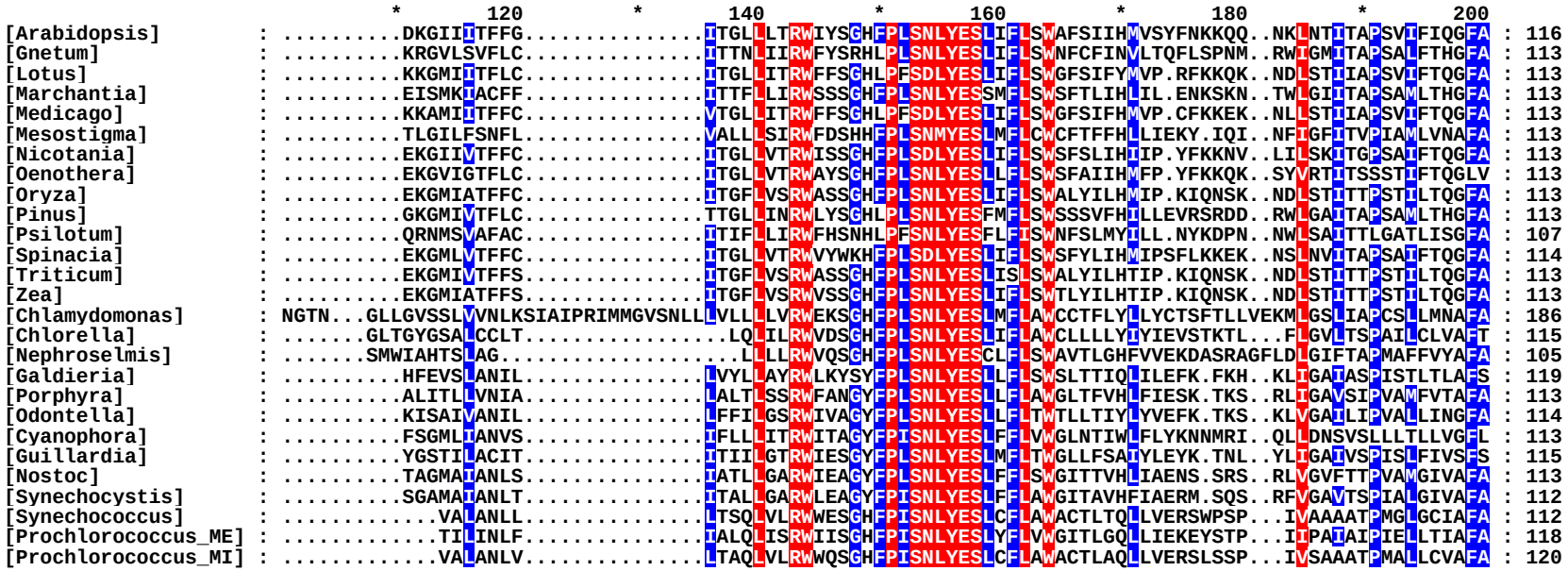

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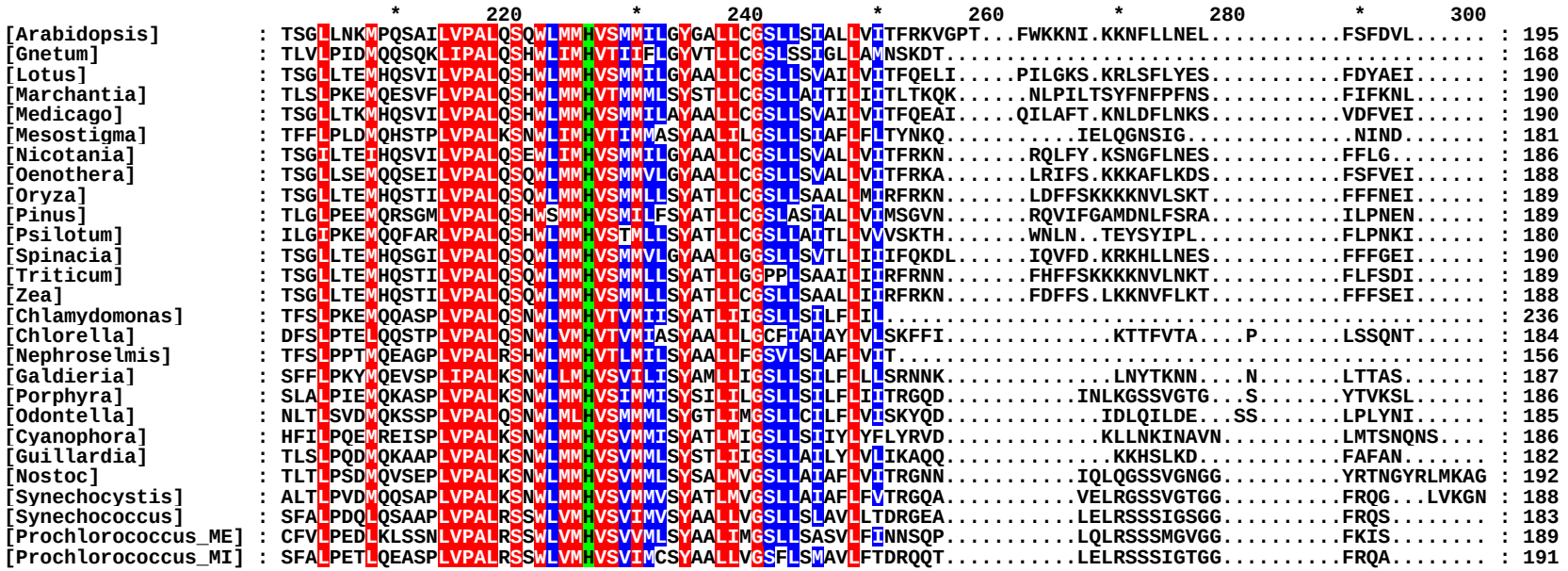

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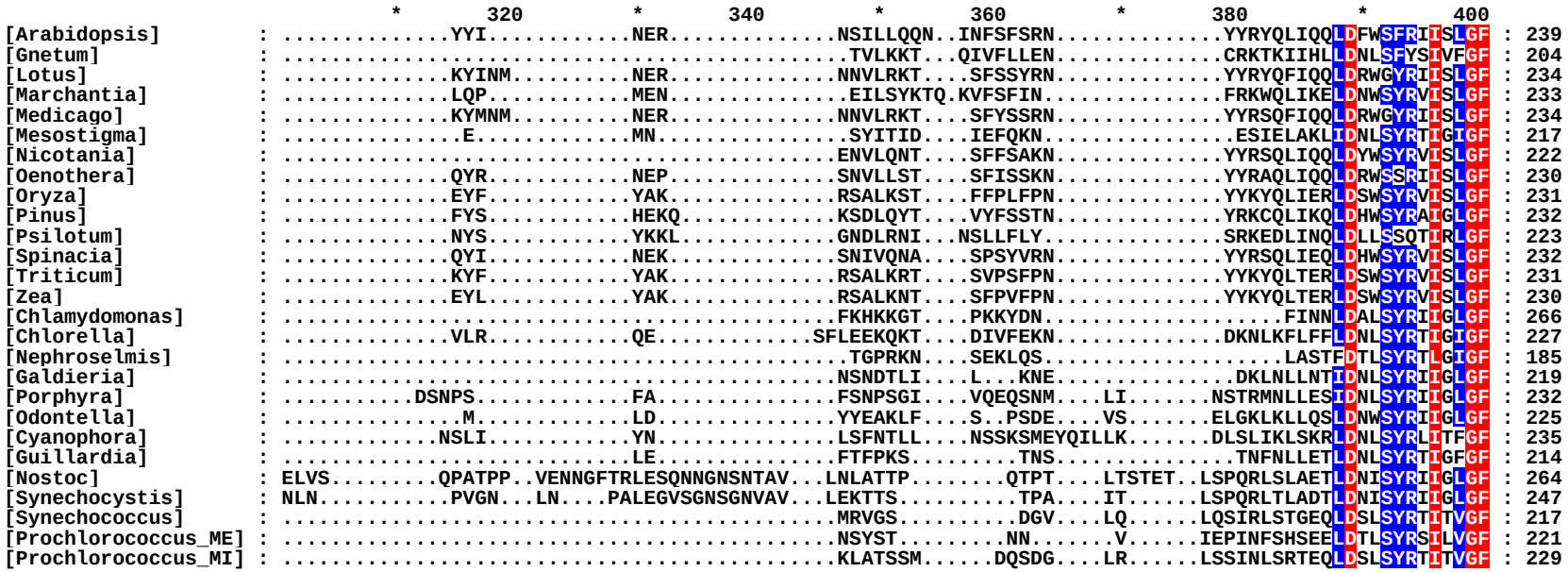

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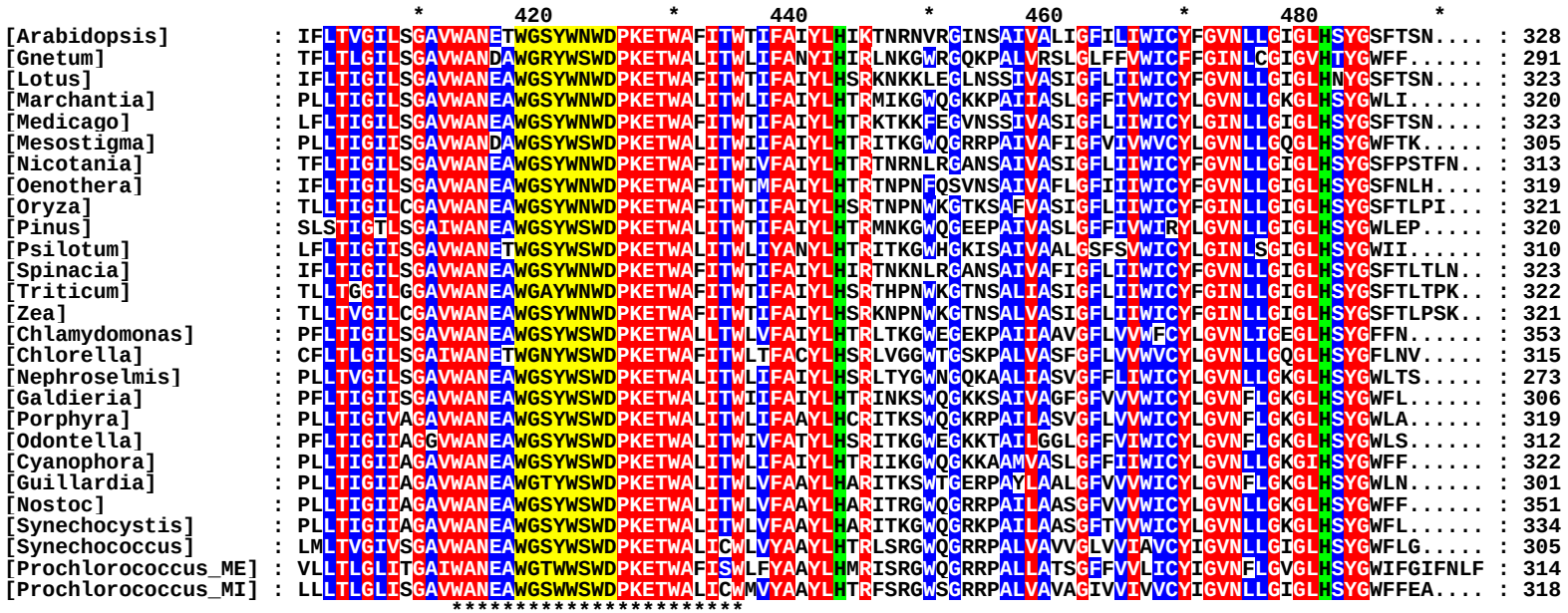

Figure Supplemental data: Conservation of CcsA in plastid and cyanobacterial genomes

CcsA homologs from plastid-encoded genomes of plants *Arabidopsis thaliana* (P56770), *Gnetum gnemon* (CAB96962), *Lotus japonicus* (NP_084844), *Marchantia polymorpha* (NP_039347), *Medicago truncatula* (AC093544), *Mesostigma viride* (Q9MUM3), *Nicotania tabacum* (P12216), *Oenothera hookeri* (Q9MTI2), *Oryza sativa* (1606356CW), *Pinus thunbergii* (BAA04448), *Psilotum nudum* (AP004638), *Spinacia oleracea* (NP_054987), *Triticum aestivum* (NP_114307), *Zea mays* (P46659), green algae *Chlamydomonas reinhardtii* (T07998), *Chorella vulgaris* (BAA57962), *Nephroselmis olivacea* (NP_050913), red algae *Galdiera sulfuraria* (P31564), *Porphyra purpurea* (NP_053979), brown alga *Odontella sinensis* (NP_043583), glaucophyte alga *Cyanophora paradoxa* (NP_043267), cryptophyte alga *Guillardia theta* (CAA36413), and from cyanobacterial genomes *Nostoc* sp. PCC 7120 (NP_484979), *Synechocystis* sp. PCC 6803 (CAA96562), *Synechococcus* sp. WH1802, *Prochlorococcus marinus* MED4 and *Prochlorococcus marinus* MIT9313 (see draft genomes for the three latter sequences at http://www.jgi.doe.gov/JGI_microbial/html/index.html) were aligned using CLUSTALW algorithm (Blosum62 scoring matrix) in Bioedit software. The alignment was edited in the GeneDoc multiple alignment editor software. Amino-acids strictly conserved in all sequences are shaded red and those conserved in the majority of the sequences (14 of 27) are shaded blue. The three conserved histidinyl residues are highlighted in green. The 23 amino-acid tryptophan rich region is indicated (*****) and includes the WGXϕWXWD signature motif highlighted in yellow. The sequence of plastid CcsA from the red alga *Cyanidium caldarium* (AF022186) is not shown on this alignment as we believe there is a frameshift error in the 3' end of the sequence which results in an early stop at codon 293 of the predicted ORF. This eliminates the C-terminal part of CcsA polypeptide where the third invariant histidine lies.