

A Novel Component of the Disulfide-Reducing Pathway Required for Cytochrome *c* Assembly in Plastids

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ABSTRACT

In plastids, the conversion of energy in the form of light to ATP requires key electron shuttles, the *c*-type cytochromes, which are defined by the covalent attachment of heme to a CXXCH motif. Plastid *c*-type cytochrome biogenesis occurs in the thylakoid lumen and requires a system for transmembrane transfer of reductants. Previously, CCDA and CCS5/HCF164, found in all plastid-containing organisms, have been proposed as two components of the disulfide-reducing pathway. In this work, we identify a small novel protein, CCS4, as a third component in this pathway. CCS4 was genetically identified in the green alga *Chlamydomonas reinhardtii* on the basis of the rescue of the *ccs4* mutant, which is blocked in the synthesis of holoforms of plastid *c*-type cytochromes, namely cytochromes *f* and *c₆*. Although CCS4 does not display sequence motifs suggestive of redox or heme-binding function, biochemical and genetic complementation experiments suggest a role in the disulfide-reducing pathway required for heme attachment to apoforms of cytochromes *c*. Exogenous thiols partially rescue the growth phenotype of the *ccs4* mutant concomitant with recovery of holocytochrome *f* accumulation, as does expression of an ectopic copy of the CCDA gene, encoding a *trans*-thylakoid transporter of reducing equivalents. We suggest that CCS4 might function to stabilize CCDA or regulate its activity.

CYTOCHROMES *c* are ubiquitous molecules functioning as electron carriers. They carry a heme cofactor covalently attached via two thioether linkages between the vinyl groups of heme B (iron protoporphyrin IX) and the cysteine sulfhydryls in the apocytochrome *c* (THÖNY-MEYER 1997). The cysteine sulfhydryls are found in a CXXCH motif, also referred to as the heme-binding site, where histidine acts as one of the axial ligands of heme. CXXCK, CXXXCH, or CXXXXCH motifs are variations to the canonical heme-binding site and are found in some bacterial cytochromes *c* (JUNGST *et al.* 1991; RIOS-VELAZQUEZ *et al.* 2001; HARTSHORNE *et al.* 2006). Another variation is found in trypanosomatid where heme is attached via a single thioether bond at a F/AXXCH motif on mitochondrial *c*-type cytochromes (ALLEN *et al.* 2004).

Bacterial cytochromes *c* are assembled in the periplasm via two different pathways, system I and system II (FERGUSON *et al.* 2008; HAMEL *et al.* 2009; KRANZ *et al.* 2009; BONNARD *et al.* 2010; SANDERS *et al.* 2010). A thiol-disulfide membrane transporter of the DsbD/CcdA

family and a membrane-anchored, periplasm-facing thioredoxin-like protein (CcmG in system I or ResA/CcsX in system II) are the defining components of the disulfide-reducing pathway. They are postulated to act sequentially to reduce the disulfide-bonded CXXCH in apocytochrome *c* prior to the heme ligation (RITZ and BECKWITH 2001; ALLEN *et al.* 2003; KADOKURA *et al.* 2003; MAPLLER and HEDERSTEDT 2006). The need for disulfide reduction in cytochrome *c* assembly is thought to be necessary because the periplasm is also the compartment where disulfide bond formation takes place (MAPLLER and HEDERSTEDT 2006; MESSENS and COLLET 2006; KADOKURA and BECKWITH 2010). Inactivation of the disulfide-reducing pathway in bacteria results in a cytochrome *c*-deficient phenotype, and it is believed that the apocytochrome *c* CXXCH then becomes the target of the disulfide bond machinery (DESHMUKH *et al.* 2000; ERLENDSSON and HEDERSTEDT 2002; TURKARSLAN *et al.* 2008).

In photosynthetic eukaryotes, *c*-type cytochromes are housed in the thylakoid lumen of plastids. Plastid cytochromes *c* are assembled through a multi-component pathway uncovered in the green alga *Chlamydomonas reinhardtii* through genetic analysis of the *ccs* mutants (*ccs* for cytochrome *c* synthesis) (HOWE and MERCHANT 1992; HOWE *et al.* 1995; XIE *et al.* 1998). These mutants are deficient for membrane-bound cytochrome *f* and

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soluble cytochrome c_6 , the two c -type cytochromes required for photosynthesis (HOWE and MERCHANT 1992). In *Chlamydomonas*, cytochrome f and cytochrome c_6 are synthesized in the plastid and cytosol, respectively. The heme attachment takes place in the thylakoid lumen, a compartment topologically analogous to the bacterial periplasm. Pulse-chase analyses in the *ccs* mutants revealed that apoforms of cytochrome f and c_6 are synthesized and further processed in the thylakoid lumen, but not converted to their respective holoforms. This indicates that the *CCS* loci control the heme attachment reaction (HOWE and MERCHANT 1992; HOWE *et al.* 1995; XIE *et al.* 1998). The *CCS* loci do not control the covalent attachment of heme Ci to cytochrome b_6 of the cytochrome b_6f complex (STROEBEL *et al.* 2003). While heme attachment via the *CCS* pathway occurs in the lumen, covalent linkage of heme Ci to a cysteine on cytochrome b_6 is dependent upon the Cofactor binding, Cytochrome b_6f complex, and subunit petB (*CCB*) factors and takes place on the stromal side of the thylakoid membrane (KURAS *et al.* 1997, 2007; LYSKA *et al.* 2007; LEZHNEVA *et al.* 2008; SAINT-MARCOUX *et al.* 2009).

The operation of a disulfide-reducing pathway in the context of plastid cytochrome c assembly was first suspected because of the occurrence of orthologs of the bacterial thiol transporter *CCDA* that localize to the plastid (NAKAMOTO *et al.* 2000; PAGE *et al.* 2004). In *Arabidopsis thaliana*, loss of *CCDA* impacts photosynthesis and results in a cytochrome b_6f assembly defect (PAGE *et al.* 2004). However, evidence that heme attachment to apocytochrome f is impaired by *ccda* mutations is still lacking, and the placement of *CCDA* in plastid cytochrome c maturation needs to be confirmed (PAGE *et al.* 2004). The finding that the *Chlamydomonas ccs4* and *ccs5* mutants could be rescued by application of exogenous thiols led to the proposal that the corresponding gene products are components of the disulfide-reducing pathway (PAGE *et al.* 2004). *CCS5*, a new locus controlling plastid cytochrome c assembly, was recently identified and shown to encode the algal ortholog of *Arabidopsis* HCF164. HCF164 is a membrane-anchored, lumen-facing thioredoxin-like protein required for cytochrome b_6f assembly (LENNARTZ *et al.* 2001; GABILLY *et al.* 2010). The recombinant form of *CCS5*/HCF164 can reduce a disulfide at the CXXCH motif of apocytochrome f (LENNARTZ *et al.* 2001; MOTOHASHI and HISABORI 2006; GABILLY *et al.* 2010).

In this article, we report the molecular identification of the *CCS4* gene by functional complementation of the *ccs4* mutant. *CCS4* does not carry any motif indicative of redox chemistry despite the fact that thiol-dependent, partial rescue of *ccs4* suggests its involvement in the reducing pathway. Expression of an ectopic copy of the *CCDA* gene, encoding the plastid thiol-disulfide transporter, partially suppresses the *ccs4* mutant. This indicates that *CCS4* and *CCDA* interact in the same redox pathway.

We discuss the possible roles of *CCS4* in the disulfide-reducing pathway required for cytochrome c maturation.

MATERIALS AND METHODS

Strains and culture conditions: The *ccs4-F2D8* mutant strain (*mt⁻*) (XIE *et al.* 1998) was crossed to a wild-type strain (*mt⁺ arg7-8*) to generate the *ccs4-F2D8 arg7-8* (*mt⁺*) used in the complementation experiments. For the thiol rescue experiments, the *ccs4-F2D8 arg7-8* strain was crossed to CC-2677 (*cw₁₅ nit1 mt⁻*) and a *cw₁₅ ccs4* strain was identified. Wild-type strains were CC124 and CC2677. Strains were grown at 22–25° in tris acetate phosphate (TAP) liquid or solid medium (HARRIS 1989) with or without copper supplementation under dim light (25 $\mu\text{mol}/\text{m}^2/\text{sec}$) for *ccs4* and *ccs5* strains or under standard illumination for wild-type strains (300 $\mu\text{mol}/\text{m}^2/\text{sec}$) as described in HOWE and MERCHANT (1992). Copper-deficient media are used to induce the expression of cytochrome c_6 (QUINN and MERCHANT 1998).

Molecular cloning of the *CCS4* gene: The *ccs4-F2D8 arg7-8* strain was transformed by electroporation using the indexed cosmid library, and phototrophic transformants were recovered on minimal medium under high light (300 $\mu\text{mol}/\text{m}^2/\text{sec}$). An 8-kb *Bam*HI fragment and a 1-kb *Sac*II fragment containing the *CCS4* gene were isolated from a complementing cosmid and cloned in pBluescript SK vector yielding the pSK-*CCS4 Bam*HI and pSK-*CCS4 Sac*II plasmids, respectively. The coding sequence of *CCS4* (from ATG to stop) was cloned at *Eco*RI and *Xba*I sites of pSL18 (POLLOCK *et al.* 2004) between the *PSAD* promoter and terminator using P*ccs4*-ORF2-*Nde*I (5'-AACCCA TATGTCGACTGGCATTGAGG-3') and L-P*ccs4*-ORF2-*Xba*I (5'-AACTCTAGATCACTTGGTTGCTGC-3') as primers and pSK-*CCS4 Sac*II as a template. The resulting plasmid is pSL18-*CCS4*(ORF1). The coding sequence corresponding to the truncated form of *CCS4* (from M32 to stop) was cloned at *Eco*RI and *Xba*I sites between the *PSAD* promoter and terminator of pSL18 via in-fusion technology (Clontech) using PORF5-F-*Eco*RI (5'-CGATAAGCTTGATATCGAATTCATGCTATTTCAAAAGG CATTGAGG-3') and PORF5-R-*Xba*I (5'-GGTCCAGCTGCTGC CATCTAGATCACTTGGTTGCTGCTCTGG-3') as primers and pSK-*CCS4 Sac*II as a template. The final construct is pSL18-*CCS4* (ORF2).

Construction of *CCDA*-expressing plasmid: The *CCDA* ORF was cloned between the *PSAD* promoter and terminator of pSL18. The cloned cDNA (NAKAMOTO *et al.* 2000) was used as a template in a PCR reaction with *ccdA-Nde*I (5'-GGGAATTC CATATGCGAACC GCCATGCATTTAG-3') and *ccdA-Eco*RI (5'-CGGAATTCTCAGAGGGCACCAGGCGCG-3') as *Nde*I- and *Eco*RI-engineered primers, respectively. The PCR product was cloned at the *Nde*I and *Eco*RI sites and yielded pSL18-*CCDA*.

RNA extraction and real time PCR: Wild-type CC124, mutant strains *ccs4-F2D8*, or *ccs4-F2D8 arg7-8* transformed with the empty cosmid pCB412 or cotransformed with pCB412 and pSK-*CCS4 Sac*II (*ccs4-Sac*) or with pCB412 and pSK-*CCS4 Bam*HI (*ccs4-Bam*) were grown in TAP medium at 25° with 25 $\mu\text{mol}/\text{m}^2/\text{sec}$ of light. At $\sim 6 \times 10^6$ cells/ml, total RNA from triplicate cultures per strain was prepared as in QUINN and MERCHANT (1998). Samples were prepared and real time PCR was performed as in ALLEN *et al.* (2007). Gene-specific primers used for amplification were 5'-GCTTCCTCCCTGCAGCCGT CCT-3' and 5'-GCGGGATCAAGCAGCGACAAGT-3' for *CCS1*; 5'-TGGTTGCCTGCTCCTTGGAC-3' and 5'-GCACGGGCTC AGATGAATGG-3' for *CCS4*; and 5'-GCGGGTTCGAGAGGT TATGG-3' and 5'-CCCTCGTCAGCCCTCTGTGT-3' for *CCDA*. Primer efficiencies for *CCS1*, *CCS4*, and *CCDA* were 102, 100, and 99%, respectively. All data were analyzed together with

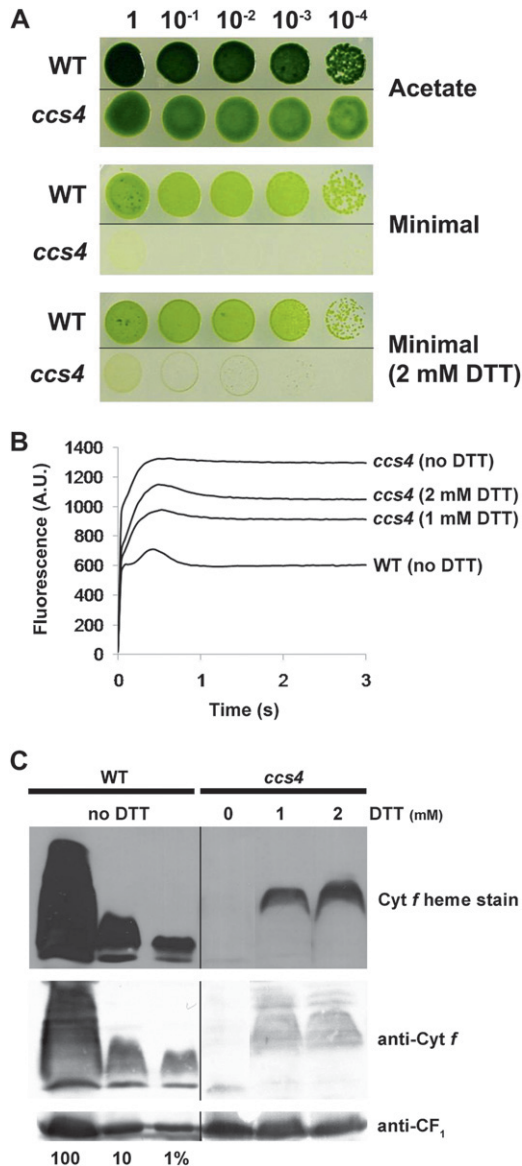


FIGURE 1.—The *ccs4* mutant is partially rescued by exogenous thiols. (A) DTT-dependent photosynthetic rescue of *ccs4*. Ten-fold dilution series of wild type (*cw15 nit1-305*) (WT) and *ccs4* (*ccs4-F2D8 cw15 arg7-8*) (*ccs4*) were plated on acetate and minimal medium with or without 2 mM DTT. Cells grown heterotrophically were incubated at 25° for 7 days with 20 $\mu\text{mol}/\text{m}^2/\text{sec}$ of light. Cells grown phototrophically with or without DTT were incubated at 25° for 14 days with 250 $\mu\text{mol}/\text{m}^2/\text{sec}$ of light. Cells grown phototrophically showed the best rescue with 2 mM DTT. (B) Fluorescence kinetics indicate a partial restoration of cytochrome b_6f in DTT-treated *ccs4* cells. The fluorescence induction and decay kinetics observed in a dark-to-light transition of *ccs4* grown in the absence or presence of 1 and 2 mM DTT are shown compared to those of WT. Fluorescence transients were measured using Handy Fluorcam from Photon System Instruments. The fluorescence is in arbitrary units (A.U.) and recorded over a 3-sec illumination period. The rise and plateau curve for *ccs4* is a signature of a specific block in electron transfer at the level of the cytochrome b_6f complex because of its impaired assembly in the absence of membrane-bound holocytochrome *f*. When the energy absorbed by the chlorophyll cannot be utilized because of a block in photosynthetic transfer through cyto-

chrome b_6f , an increase in the chlorophyll fluorescence is observed. In wild type, the decay phase corresponds to the re-oxidation of the quinone pool, the primary electron acceptor of the photosystem II, by the cytochrome b_6f complex. (C) DTT-dependent partial restoration of holocytochrome *f* assembly in *ccs4*. Cytochrome *f* heme staining and anti-cytochrome *f* immunoblot analyses were performed on total protein extracts from *ccs4* (*ccs4-F2D8 cw15 arg7-8*) and wild-type (*cw15*) strains. Cells were grown heterotrophically (on acetate and in low light) in the absence or presence of 1 or 2 mM DTT. Samples of WT and *ccs4* strains corresponding to 18 μg of chlorophyll were separated in SDS-containing acrylamide (12%) gel. The gel was then transferred to a PVDF membrane to perform heme staining and immunodecoration with antisera against cytochrome *f* and CF_1 of the ATPase that serves as a loading control. Dilutions of the wild-type sample serve to estimate the cytochrome *f* abundance.

LinRegPCR 11.x to obtain the mean PCR efficiency for each gene (RUIJTER *et al.* 2009). Transcript levels for the genes of interest (*gt*) were normalized to the transcript levels of the *CBLP* gene encoding the Chlamydomonas β -subunit-like polypeptide. Relative transcript level (RTL) was calculated as follows: $\text{RTL} = 1000 \times [\text{mean PCR efficiency for } CBLP]^{C_{CBLP}} \times [\text{mean PCR efficiency for } gt]^{C_{gt}}$.

Protein preparation and analysis: Supernatant and pellet fractions were obtained by freeze-thaw fractionation and subsequent centrifugation. Fractions were electrophoretically separated and cytochromes *c* were revealed by immunodetection or by a heme-staining procedure (HOWE and MERCHANT 1992). Polyclonal antisera raised against Chlamydomonas cytochrome c_6 , cytochrome *f* GST-fusion protein, CCS5, CF_1 , and plastocyanin were used for immunodetection by alkaline phosphatase-conjugated secondary antibodies.

RESULTS

CCS4 gene product may participate in disulfide reduction: On the basis of our understanding of the biochemistry of cytochrome *c* maturation, it is expected that some of the *CCS* loci control disulfide reduction. In an attempt to functionally categorize the gene products corresponding to the genetically defined *ccs* mutants, we tested for the rescue of the *ccs4* mutant by exogenous thiols. Our approach is driven by precedence in bacteria (BECKETT *et al.* 2000; DESHMUKH *et al.* 2000; BARDISCHEWSKY and FRIEDRICH 2001; ERLENDSSON and HEDERSTEDT 2002; FEISSNER *et al.* 2005). Moreover, we have shown that *ccs5* could be rescued by DTT (PAGE *et al.* 2004; GABILLY *et al.* 2010). As shown in Figure 1A, addition of DTT to minimal medium can rescue the photosynthetic deficiency of *ccs4*. We noted that 2-mercaptoethane sulfonate, a reduced thiol, is also able to rescue *ccs4* to the same extent as DTT (data not shown). This partial rescue is dose dependent and correlates with a restoration of the cytochrome b_6f function, as evidenced by fluorescence rise and decay kinetics (Figure 1B). Heme stain and immunoblot analysis confirmed that levels of holocytochrome *f* are increased in DTT-treated cells (Figure 1C). Consistent with the partial restoration of the photosynthetic

chrome b_6f , an increase in the chlorophyll fluorescence is observed. In wild type, the decay phase corresponds to the re-oxidation of the quinone pool, the primary electron acceptor of the photosystem II, by the cytochrome b_6f complex. (C) DTT-dependent partial restoration of holocytochrome *f* assembly in *ccs4*. Cytochrome *f* heme staining and anti-cytochrome *f* immunoblot analyses were performed on total protein extracts from *ccs4* (*ccs4-F2D8 cw15 arg7-8*) and wild-type (*cw15*) strains. Cells were grown heterotrophically (on acetate and in low light) in the absence or presence of 1 or 2 mM DTT. Samples of WT and *ccs4* strains corresponding to 18 μg of chlorophyll were separated in SDS-containing acrylamide (12%) gel. The gel was then transferred to a PVDF membrane to perform heme staining and immunodecoration with antisera against cytochrome *f* and CF_1 of the ATPase that serves as a loading control. Dilutions of the wild-type sample serve to estimate the cytochrome *f* abundance.

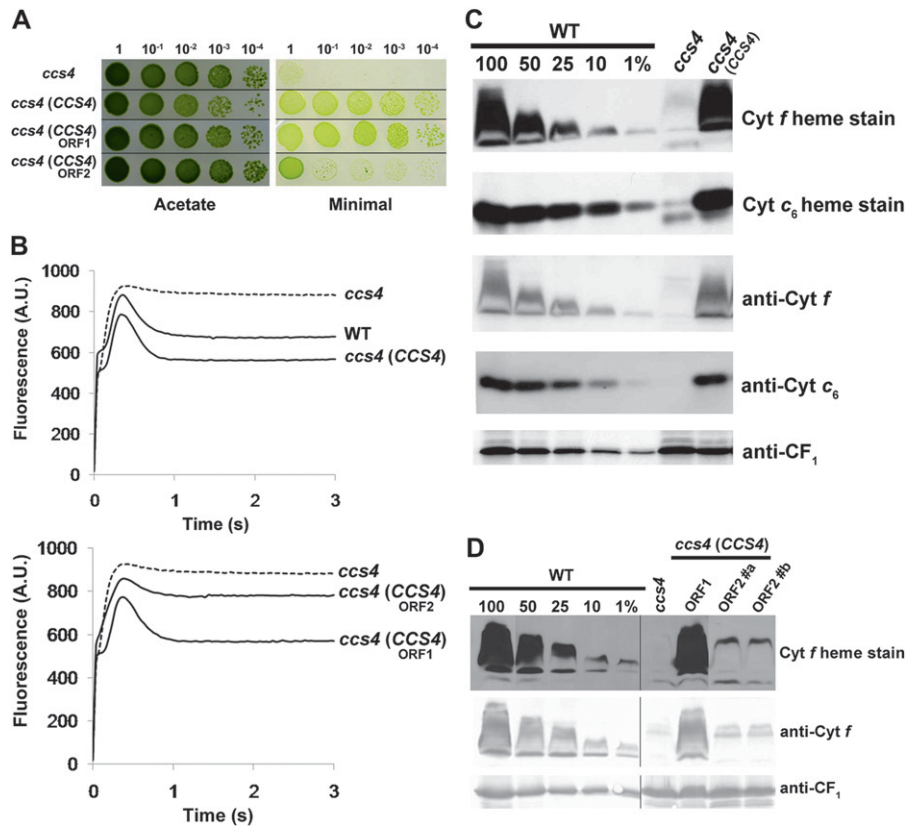


FIGURE 2.—Complementation of the *ccs4* mutant. (A, B, and D) The *ccs4-F2D8 arg7-8* strain was transformed with pSL18 (*ccs4*), pSL18 carrying a 1-kb genomic fragment with the *CCS4* gene (*CCS4*), and pSL18 expressing the full-length *CCS4* coding sequence (*CCS4*, ORF1) or a truncated form of the *CCS4* protein (*CCS4*, ORF2). (C) The *ccs4-F2D8 arg7-8* strain was transformed with pCB412 (*ccs4*) or cotransformed with pCB412 and pSK-*CCS4*-*SadI* carrying a 1-kb genomic fragment with the *CCS4* gene [*ccs4* (*CCS4*)]. Only one representative transformant is shown in A–C. In D, two representative transformants (*CCS4*, ORF2) are shown. In B–D, CC124 is the wild-type strain (WT). (A) Restoration of the photosynthetic growth of *ccs4* by full-length and truncated *CCS4*. Ten-fold dilution series of each transformant were plated on acetate (under heterotrophic conditions, 20 $\mu\text{mol}/\text{m}^2/\text{sec}$ of light) and minimal medium (phototrophic conditions, 250 $\mu\text{mol}/\text{m}^2/\text{sec}$ of light) and incubated at 25° for 1 and 3 weeks, respectively. (B) Fluorescence kinetics indicate restoration of cytochrome *b₆f* in *ccs4* complemented with the full-length and truncated *CCS4* gene. Fluorescence transients were measured on colonies

grown for 1 day on solid acetate medium after a short dark adaptation using Handy Fluorcam (Photon System Instruments). The fluorescence is in arbitrary units (A.U.) and recorded over a 3-sec illumination period. (C) Plastid *c*-type cytochromes accumulation is restored in *ccs4* complemented with the *CCS4* gene. Strains were analyzed for cytochrome *f* and cytochrome *c₆* accumulation by heme stain and immunoblot. Samples corresponding to 18 μg of chlorophyll were separated in 12% SDS acrylamide gel to detect cytochrome *f* and *CF₁* that serves as loading control. Samples corresponding to 16 μg of chlorophyll were separated in 15% native acrylamide gel to detect cytochrome *c₆*. For an estimation of the protein abundance in the *ccs4*-complemented strain, dilutions of the wild-type sample were loaded on the gel. Gels were transferred to PVDF membranes prior to heme staining and immunodetection with antisera against cytochrome *f*, cytochrome *c₆*, and *CF₁*. (D) Cytochrome *f* accumulation is partially restored in *ccs4* complemented by a truncated form of the *CCS4* gene. Strains were analyzed for cytochrome *f* accumulation via heme stain and immunoblot. Experimental conditions are the same as described in C.

growth, accumulation of holocytochrome *f* is only marginally increased in DTT-treated *ccs4* cells.

These results indicate that the *CCS4* gene product may participate in disulfide reduction. We have ruled out the possibility that the *CCS4* gene encodes for *CCDA* because the *CCDA* locus was intact in the *ccs4* mutant (PAGE *et al.* 2004). Because *CCS4* is genetically distinct from the *CCS5* locus (PAGE *et al.* 2004; GABILLY *et al.* 2010), we concluded that *CCS4* must encode a novel redox component involved in cytochrome *c* maturation.

Cloning of the *CCS4* gene by functional complementation of the *ccs4-F2D8* mutant: We sought to clone the *CCS4* gene by complementation of the photosynthetic deficiency of a *ccs4-F2D8 arg7-8* strain using an indexed ARG7-based cosmid library (PURTON and ROCHAIX 1994). Three cosmids with overlapping inserts were identified as restoring the photosynthetic competence when introduced into the *ccs4-F2D8 arg7* strain (not shown). The complementing activity could be isolated to a 1-kb *SadI* fragment, suggesting that the *CCS4* gene

is very small (Figure 2A). This 1-kb fragment restored photosynthetic growth (Figure 2A), fluorescence rise, and decay kinetics, indicating that the cytochrome *b₆f* complex is functional (Figure 2B) and the accumulation of holofoms of cytochrome *f* and *c₆* to wild-type levels (Figure 2C).

***CCS4* gene encodes a unique protein with no known motif:** RT-PCR experiments showed that the genomic region corresponding to the 1-kb *SadI* complementing fragment is transcriptionally active (not shown). However, the size of the full-length transcript could not be determined, as RNA hybridization failed to detect the mRNA, presumably because of its low abundance (not shown). A 285-bp cDNA sequence was assembled from sequencing of RT-PCR products. Interestingly, the *CCS4* pre-mRNA contains two small introns of 88 and 104 bp, a rare occurrence as most Chlamydomonas genes contain an average intron size of 373 bp (MERCHANT *et al.* 2007). Quantitative RT-PCR experiments, using primers that map to the transcript, evidenced a sixfold

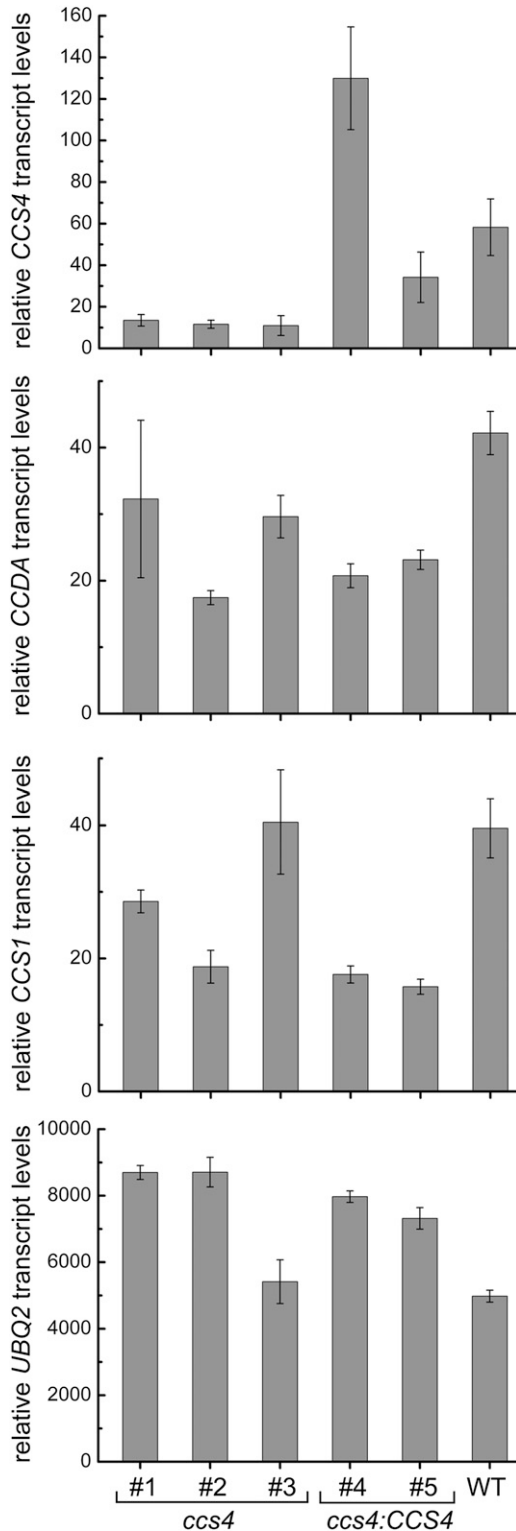


FIGURE 3.—Relative *CCS4*, *CCDA*, and *CCS1* mRNA abundance in *ccs4* and *ccs4* (*CCS4*)-complemented strains. RNA was isolated and analyzed by real time PCR. Strains are wild-type CC124 (WT), *ccs4-F2D8 arg7-8* (#1), *ccs4-F2D8 arg7-8* strain transformed by cosmid pCB412 (#2), *ccs4-F2D8* mutant (#3), *ccs4-F2D8 arg7-8* strain cotransformed by pCB412 and pSK-*CCS4* *Bam*HI (#4), or cotransformed by pCB412 and pSK-*CCS4* *Sad*II (#5). Relative transcript levels (RTLs) represent the mean levels of three independent experiments, each analyzed in technical triplicates. RTL values are relative

reduction in the accumulation of the mRNA in the *ccs4* mutant compared to the wild-type strain but increased levels in the *ccs4* strain complemented with genomic fragments containing the *CCS4* gene (Figure 3). One ORF was identified from sequencing of the RT-PCR products (Figure 4; accession no. ADL27744). This ORF encodes a 93-amino-acid protein with no motifs or residues indicative of redox (*e.g.*, cysteine) or any other biochemical activity. The predicted protein contains an N-terminal hydrophobic stretch that could serve as a membrane anchor and a C-terminal domain rich in charged residues (12 negatively charged and 9 positively charged). On the basis of the positive-inside rule that governs the topology of bacterial and thylakoid membrane proteins (VON HEIJNE 1989; GAVEL *et al.* 1991), the C-terminal domain of *CCS4* is predicted to be exposed to the stromal side of the thylakoid membrane. Standard protein targeting algorithms failed to predict plastid localization, an intriguing finding considering that we expect the protein to act in the plastid. Moreover, the only structural homologs in the database corresponded to predicted proteins in *Volvox carteri* and *Dunaliella salina*, two algae closely related to *Chlamydomonas* (HERRON *et al.* 2009; PROCHNIK *et al.* 2010). The predicted *Volvox* protein is 60% identical to its *Chlamydomonas* counterpart (Figure 4), which reflects considerable divergence compared to sequences of cytochrome assembly factors *CCS1* (INOUE *et al.* 1997), *CCDA* (NAKAMOTO *et al.* 2000), and *CCB1* (KURAS *et al.* 2007), which are 79, 83, and 85% identical, respectively. The fact that *Volvox* *CCS4* has diverged from its *Chlamydomonas* counterpart indicates that *CCS4*-like proteins might not be easily recognizable on the basis of sequence similarity in other photosynthetic eukaryotes. Sequencing of the 1-kb *Sad*II genomic fragment in the *ccs4* mutant strain identified one molecular lesion (C to T) in the coding region of the *CCS4* gene. This change results in a nonsense mutation at residue Q₅₀ in the predicted sequence (CAG to TAG) and presumably produces a nonfunctional truncated protein (Figure 4). To ascertain that we identified the correct ORF for the *CCS4* gene, we cloned the genomic sequence from ATG (M1) to stop (ORF1) in an expression vector (pSL18) containing a paromomycin-resistance (*Pm*^R) cassette as a selectable marker. The resulting construct (pSL18/*CCS4*-ORF1) was introduced in the *ccs4* mutant. Of 22 *Pm*^R transformants, 12 were able to grow photosynthetically and displayed wild-type fluorescence rise and decay kinetics (Figure 2, A and B). As expected from the restoration of the photosynthetic growth, cytochrome *f* assembly is also restored to wild-type levels (Figure 2D). The level of complementation is identical to that of the transformants carrying the 1-kb

to the *CBLP* levels and were calculated as described in MATERIALS AND METHODS. The abundance of *UBQ2* is shown as a control.

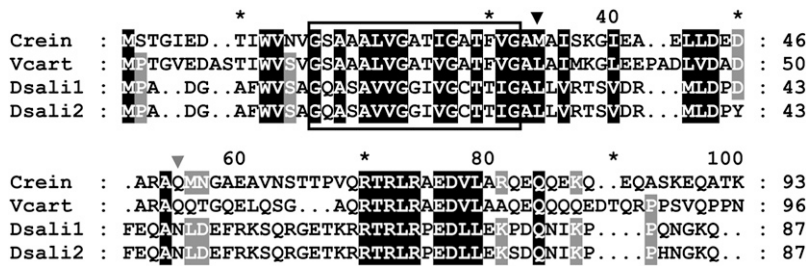


FIGURE 4.—Alignment of *Chlamydomonas*, *Volvox*, and *Dunaliella* CCS4 proteins. Sequences of *C. reinhardtii* (Crein, accession no. ADL27744), *V. carteri* (Vcart, accession no. FD920844.1), *D. salina* (Dsali1, accession no. BM447122.1; Dsali2, accession no. BM448413.1) CCS4 were aligned using the CLUSTALW algorithm (Blosum62 scoring matrix) in Bioedit. The alignment was edited using the GeneDoc multiple alignment editor. Strictly conserved or similar amino acids are on a solid background. The putative membrane anchor is boxed. The solid downward arrowhead indicates the position of the methionine in the truncated CCS4 form and the shaded downward arrowhead indicates the Q residue that is mutated to a stop codon in the *ccs4-F2D8* strain.

anchor is boxed. The solid downward arrowhead indicates the position of the methionine in the truncated CCS4 form and the shaded downward arrowhead indicates the Q residue that is mutated to a stop codon in the *ccs4-F2D8* strain.

SadI genomic fragment, suggesting that ORF1 encodes the *CCS4* gene product (Figure 2, compare A, B and C, D). To determine which of the two methionines (M1 and M32) serve as an initiation codon (Figure 4), we performed site-directed mutagenesis and tested the ability of the mutant forms to complement the *ccs4* mutation. Mutagenesis of M1 abolished complementation while alteration of M32 did not (not shown). This confirms that M1 is the initiation codon of the *CCS4* gene. We took advantage of the presence of the second methionine (M32) to generate a modified version of the *CCS4* gene expressing a truncated form of the CCS4 protein, missing the first 31 amino acids, including the predicted transmembrane domain (Figure 4). We cloned the truncated sequence from ATG to stop (ORF2) in the same expression vector used for our complementation experiments. Of 103 Pm^R transformants, 43 exhibited partial complementation of the photosynthetic growth defect and pseudo-wild-type fluorescence rise and decay kinetics (Figure 2, A and B). Enhanced levels of cytochrome *f* accumulated in the partially rescued transformants compared to the *ccs4* mutant strain, suggesting that the truncated form of the CCS4 protein retained some activity (Figure 2D). Note that the level of holocytochrome *f* restoration upon expression of the truncated *CCS4* gene is similar to that observed in the DTT-rescued *ccs4* cells (Figure 1C). As a control, we showed that transformation of the *ccs4* mutant with the empty plasmid yielded no photosynthetic clones among 98 Pm^R transformants tested. This ruled out the possibility that the partial rescue depended upon the genomic site of integration or was caused by reversion of the photosynthetic deficiency. Unfortunately, despite several attempts, we could not generate a functional tagged version of the *CCS4* gene to assess the localization of the gene product within the cell.

Genetic interaction with *CCDA* indicates the involvement of CCS4 in the disulfide-reducing pathway: The thiol-based rescue of the *ccs4* mutant is an intriguing finding, considering that the CCS4 protein does not display any motif or cysteine residue to indicate reducing activity. We reasoned the thiol-based rescue of the *ccs4* mutant must be indirect, operating via redox components interacting with CCS4. One possible scenario is that the *ccs4* mutation inactivates the transfer of

reducing equivalents to the thylakoid lumen. In plastids, this transfer requires the activity of thiol-disulfide transporter CCDA and thioredoxin-like CCS5/HCF164 (PAGE *et al.* 2004; MOTOHASHI and HISABORI 2006; GABILLY *et al.* 2010; MOTOHASHI and HISABORI 2010). Real time PCR experiments showed no reduction in the abundance of the *CCDA* and *CCS5* transcripts in response to the *ccs4* mutation (Figure 3; data not shown). Therefore, we do not envision CCS4 as a regulator of the expression of either *CCS5* or *CCDA*. Nevertheless, an impact on the abundance of the corresponding polypeptides is a possibility. We could not test the abundance of the CCDA protein in *ccs4* because of the lack of antibodies, but immunoblot analyses with an anti-CCS5 antibody (GABILLY *et al.* 2010) showed that the level of CCS5 is unchanged in the *ccs4* mutant (Figure 5).

In parallel work, we noted that *ccs4* could be rescued by plasmids carrying the promoter-less, full-length *CCDA* cDNA when we selected for phototrophic colonies following transformation of *ccs4* (not shown). The frequency was low, but because recovery of photosynthetic proficiency was linked to the introduced cDNA in the rescued strain, the observation was genuine. We reasoned that such transformants must arise from integration of the *CCDA* cDNA in the vicinity of a promoter and therefore were few in number. This suggests that photosynthetic rescue of the *ccs4* mutation would occur only upon increased expression of the *CCDA* transcript. To test this, we cloned the *CCDA* cDNA (from ATG to stop) in front of the *PSAD* promoter,

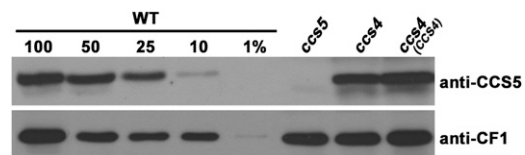


FIGURE 5.—Accumulation of the CCS5 protein in *ccs4*. Total protein (corresponding to 20 μ g of chlorophyll) from wild-type CC124 (WT), T78.15b⁻ (*ccs5*), *ccs4-F2D8 arg7-8* mutant (*ccs4*), and *ccs4-F2D8 arg7-8* complemented with pSL18-CCS4 (ORF1) (*CCS4*) was analyzed by SDS-PAGE (12%) and immunoblotting with antisera against CCS5 or CF₁ of the ATPase that serves as a loading control. For an estimation of the protein abundance, dilutions of the wild-type sample were loaded on the gel.

previously used in *Chlamydomonas* to drive expression of cDNAs (FISCHER and ROCHAIX 2001). The *CCDA*-expressing construct (pSL18/*CCDA*) was introduced in the *ccs4* mutant and transformants were selected on the basis of their resistance to paromomycin, a trait conferred by the selectable marker on the construct. Of 44 Pm^R transformants, 14 were considered as suppressed for the CCS phenotype on the basis of the partial restoration of the photosynthetic growth defect (Figure 6A). While 12 transformants were weakly suppressed, 2 displayed an increased level of phototrophic growth (Figure 6A). To confirm our results, we cotransformed the *ccs4* mutant with the Pm^R cassette containing vector pSL18 and a plasmid containing only the full-length *CCDA* cDNA (lacking promoter and terminator sequences for expression). Of 45 Pm^R transformants, 15 were weakly suppressed for the photosynthetic defect while 2 displayed a stronger restoration of the photosynthetic growth. As a control, we used a construct expressing the *CCS4* gene from the same plasmid and transformed the *ccs4* mutant. Of 22 Pm^R transformants, 12 displayed photosynthetic growth and fluorescence transients indistinguishable from wild type (Figure 6, A and B). No photosynthetic transformants were obtained among the 45 Pm^R transformants generated with the empty vector pSL18, ruling out the possibility that the two classes of suppressed transformants that we recovered with pSL18/*CCDA* resulted from reversion to photosynthetic proficiency. The *CCDA*-dependent suppression correlated with partial restoration of the cytochrome *b₆f* activity, and therefore with holocytochrome *f* assembly, in the transformed strains (Figure 6B). However, we could only demonstrate enhanced holocytochrome *f* accumulation in the strongly suppressed transformants (Figure 6C). It is likely that the level of holocytochrome *f* is only marginally increased in the weakly suppressed strains and falls below the detection limit of our heme stain technique. RT-PCR experiments showed that the ectopic copy of the *CCDA* gene is expressed in both weakly and strongly suppressed transformants (supporting information, Figure S1).

The *CCDA*-dependent suppression was specific for the *ccs4* strain. When we tested the *ccs5* mutant for rescue by *CCDA*, none of the 102 transformants screened displayed a restoration of photosynthesis (not shown). Our results suggest that *CCDA* is a component in the reducing pathway for cytochrome *c* maturation and can substitute partially for loss of *CCS4* function when expressed ectopically.

DISCUSSION

In this article, we have further dissected the plastid disulfide-reducing pathway operating in cytochrome *c* assembly. We show that (1) the *ccs4* mutant is partially rescued by exogenous thiols, (2) the *CCS4* gene encodes a novel and unique protein with no motif suggestive of a

redox activity, and (3) expression of an ectopic copy of the *CCDA* gene partially suppresses the *ccs4* mutant.

A bacterial-like, *trans*-thylakoid, disulfide-reducing pathway: An indication that the *CCS4* and *CCS5* gene products participate in the disulfide-reducing pathway is inferred from the observation that reduced thiols can rescue the cytochrome *c* assembly phenotype of the *ccs4* (Figure 1) and *ccs5* mutants (GABILLY *et al.* 2010). In bacteria, the disulfide-reducing pathway is defined by a membrane thiol-disulfide transporter (DsbD/*CcdA*) and a thioredoxin-like protein (CcmG/*ResA*/*CcsX*). This pathway is postulated to transfer reducing equivalents across the membrane for reduction of the CXXCH disulfide in apocytochrome *c* prior to the covalent attachment of heme (FERGUSON *et al.* 2008; HAMEL *et al.* 2009; KRANZ *et al.* 2009; BONNARD *et al.* 2010; SANDERS *et al.* 2010). The ability of exogenous thiol compounds to bypass mutations inactivating the disulfide-reducing components (SAMBONGI and FERGUSON 1994; BECKETT *et al.* 2000; DESHMUKH *et al.* 2000; BARDISCHEWSKY and FRIEDRICH 2001; ERLENDSSON and HEDERSTEDT 2002; FEISSNER *et al.* 2005) and the fact that recombinant *ResA* and *CcsX* can participate in thiol-disulfide exchange reactions support this proposal (MONIKA *et al.* 1997; SETTERDAHL *et al.* 2000). The occurrence of *CcdA*-like proteins in plastids suggests that a *trans*-thylakoid, disulfide-reducing pathway, similar to the one found in bacteria, is required for the maturation of cytochromes *c* in the lumen (NAKAMOTO *et al.* 2000; PAGE *et al.* 2004). The first component of this pathway was discovered via the identification of the *CCS5*/*HCF164* protein, a membrane-bound, lumen-facing, thioredoxin-like protein shown to act as an apocytochrome *f* CXXCH disulfide reductase (LENNARTZ *et al.* 2001; MOTOHASHI and HISABORI 2006; GABILLY *et al.* 2010). Our finding that expression of *CCDA* is able to suppress the *ccs4* mutant solidifies the placement of the thiol-disulfide transporter in plastid cytochrome *c* maturation (Figure 6). Indeed, earlier studies in *Arabidopsis* support, but do not establish, the requirement of plastid *CCDA* in the conversion of apo- to holocytochromes *c* (PAGE *et al.* 2004). The working model is that *CCS5*/*HCF164* is maintained in a reduced state via the activity of *CCDA*, but this awaits experimental confirmation (PAGE *et al.* 2004; MOTOHASHI and HISABORI 2006; GABILLY *et al.* 2010; MOTOHASHI and HISABORI 2010). Thioredoxin-*m* was postulated as a possible reductant of *CCDA* on the stromal side based on the observation that both *CCDA* and *CCS5*/*HCF164* can be reduced in intact *Arabidopsis* thylakoids by recombinant spinach thioredoxin-*m* (MOTOHASHI and HISABORI 2006, 2010).

What is the function of the *CCS4* protein?: It is unlikely that *CCS4* has a reducing activity in the assembly process because there are no motifs and residues in the protein sequence implying such an activity (Figure 4). One possibility is that the *ccs4* mutation results in a loss of *CCDA* function. This is compatible

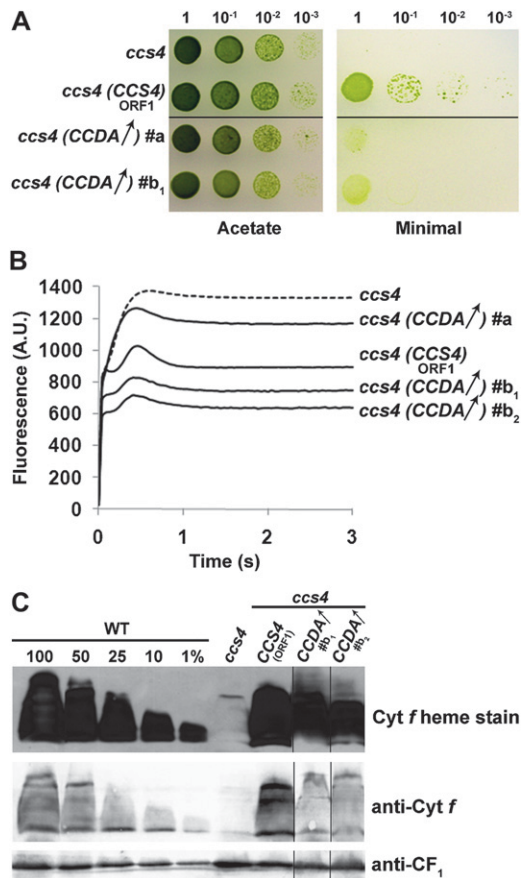


FIGURE 6.—Expression of an ectopic copy of the *CCDA* gene partially suppresses *ccs4*. The *ccs4-F2D8 arg7-8* strain was transformed with pSL18 (*ccs4*), pSL18 expressing the full-length *CCS4* coding sequence (*CCS4*, ORF1), and pSL18 expressing the *CCDA* ORF (*CCDA↑*, transformants #a and #b₁) or cotransformed with pSL18 and the *CCDA* cDNA cloned (without promoter and terminator sequences) in pBluescript (*CCDA↑*, transformant #b₂). (A) Expression of an ectopic copy of *CCDA* partially restores the phototrophic growth of *ccs4*. Ten-fold dilution series were plated on acetate (heterotrophic conditions, 20 $\mu\text{mol}/\text{m}^2/\text{sec}$ of light) and minimal medium (phototrophic conditions, 300 $\mu\text{mol}/\text{m}^2/\text{sec}$ of light) and incubated at 25° for 1 and 3 weeks, respectively. (B) Fluorescence kinetics indicate partial restoration of cytochrome *b₆f* in *ccs4* expressing an ectopic copy of *CCDA*. Fluorescence induction and decay kinetics were measured as described in Figure 1B. (C) Holocytochrome *f* accumulation is partially restored in the *ccs4* mutant expressing an ectopic copy of *CCDA*. Strains were analyzed for cytochrome *f* accumulation by heme stain and immunoblot. Experimental conditions are the same as described in Figure 2C.

with the fact that (1) *ccs4* can be partially rescued by DTT (Figure 1), as seen in bacterial *ccdA/dsbD* mutants that are restored for cytochrome *c* assembly in the presence of exogenous thiols (SAMBONGI and FERGUSON 1994; BECKETT *et al.* 2000; DESHMUKH *et al.* 2000), and (2) expression of *CCDA* can partially bypass the *ccs4* mutation (Figure 6). In one scenario, CCS4 could operate by stabilizing CCDA in the thylakoid membrane. The presence of a putative transmembrane domain in the CCS4 protein is compatible with such a hypothesis. However,

this transmembrane domain is not absolutely required for function, as a truncated form of CCS4, lacking the hydrophobic stretch, still retains some activity in the assembly of plastid cytochromes *c* (Figure 2). Another possibility is that CCS4 controls the activity of CCDA by facilitating the delivery of reducing equivalents from the stroma to the thylakoid lumen. It is conceivable that CCS4 acts as a “holdase” for presentation of the apocytochrome *c* CXXCH to the CCS5/HCF164 reductase in the thylakoid lumen. However, this model is unlikely because the positive-inside rule predicts a stromal localization for the C-terminal domain of CCS4. Moreover, a direct interaction of the CCS4 C-terminal domain with plastid apoforms of cytochromes *c* could not be detected via yeast two-hybrid using apocytochrome *f* as prey (not shown).

We could not determine a subcellular localization for CCS4; therefore, we cannot exclude that CCS4 could act in the cytosol. One possibility is that CCS4 acts as a chaperone or import factor in the cytosol. However, we find this hypothesis unlikely because the *ccs4* mutant is specifically deficient in plastid cytochromes *c* and does not display a pleiotropic phenotype. Hence, we favor a model where CCS4 is localized at the thylakoid membrane and interacts with CCDA by stabilizing the protein and/or controlling its activity, possibly via its C-terminal domain. It is conceivable that loss of CCS4 results in decreased activity and/or destabilization of CCDA, and this is consistent with expression of *CCDA* partially rescuing the phenotype. There are several examples of polytopic membrane proteins whose stability and activity are influenced by the presence of single transmembrane proteins (SCHULZ *et al.* 1999; YU *et al.* 1999; PETERS *et al.* 2008). Interestingly, in system I bacteria, CcmD, a small transmembrane protein containing a cytoplasm-facing C-terminal domain with charged residues, controls the activity of cytochrome *c* assembly factors involved in the heme relay pathway (GOLDMAN *et al.* 1997; SCHULZ *et al.* 2000; AHUJA and THÖNY-MEYER 2005; RICHARD-FOGAL *et al.* 2008). CcmD was shown to physically interact with the heme relay Ccm components and also influence their stability in the membrane (SCHULZ *et al.* 2000; AHUJA and THÖNY-MEYER 2005; RICHARD-FOGAL *et al.* 2008). Loss of CcmD can be partially rescued by overexpression of the CcmCE proteins, two key components of the heme delivery complex (SCHULZ *et al.* 1999).

Unique features of the CCS4 protein: If CCS4 acts in the plastid, its import mechanism remains to be understood as the protein does not display a typical N-terminal targeting sequence (Figure 4). Intriguingly, a truncated form of CCS4 lacking the putative transmembrane domain still retains some activity. This indicates that the putative targeting information does not lie in the N-terminal part of the protein. It is conceivable that CCS4 reaches the plastid via internal targeting signals. Recent proteomics data revealed that 20% of plastid resident proteins are devoid of N-terminal

targeting sequences and are not processed upon import in the plastid (KLEFFMANN *et al.* 2004).

The CCS4 protein does not appear to be evolutionarily conserved at the primary sequence level (Figure 4). We could not find any CCS4 orthologs in other genomes, including genomes of green algae such as *Ostreococcus* and *Chlorella*, in addition to *V. carteri* and *D. salina*. One possibility is that the function of CCS4 is dependent upon the overall charge of the protein rather than upon a specific primary sequence. The primary sequence of CcmD in bacterial system I cytochrome *c* maturation does not appear to be conserved, yet CcmD-like proteins can be recognized on the basis of charge conservation in operons containing cytochrome *c* biogenesis genes (AHUJA and THÖNY-MEYER 2005; RICHARD-FOGAL *et al.* 2008). Another possibility is that CCS4 is restricted to Volvocales, an order of green algae including *Chlamydomonas* (MERCHANT *et al.* 2007), *Dunaliella* (OREN 2005), and *Volvox* (PROCHNIK *et al.* 2010). Indeed, genomics and proteomics studies have revealed that Volvocales harbor unique proteins in their organelles (ATTEIA *et al.* 2009; PROCHNIK *et al.* 2010).

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Supporting Information

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A Novel Component of the Disulfide-Reducing Pathway Required for Cytochrome *c* Assembly in Plastids

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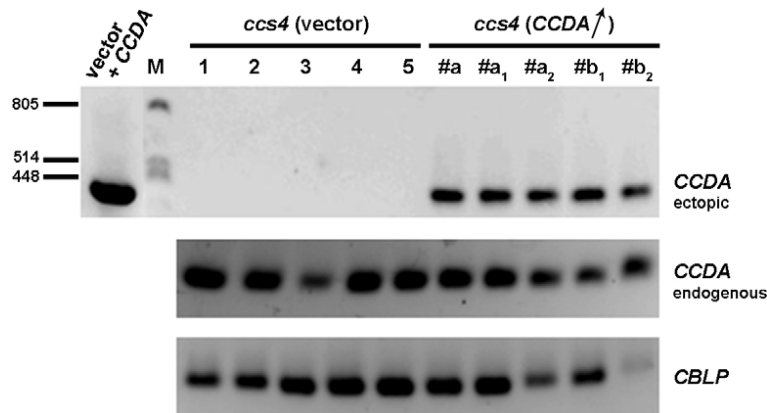


FIGURE S1.—Real-time PCR analysis was used to evaluate the expression of ectopic *CCDA*, endogenous *CCDA* and *CBLP* in the *ccs4* strain transformed by the empty pSL18 plasmid (vector) or pSL18 containing the Chlamydomonas *CCDA* cDNA (*CCDA*↑). cDNA was synthesized from total RNA and RT-PCR was performed using PCCDA-3'UTR-1 (5'-GCGGGGTCGAGAGGTTATGG-3') and PCCDA-3'UTR-2 (5'-CCCTCGTCAGCCCCTCTGTGT-3') to detect the endogenous *CCDA* transcript, PpSL18-1 (5'-CTGCTACTCACAACAAGC-3') and PCCDA1-2 (5'-ACCATTGCCGTTGTGTCCTGGCTATAC-3') to detect the ectopic *CCDA* transcript and PCBLP-1 (5'-GCCACACCGAGTGGGTGTCGTGCG-3') and PCBLP-2 (5'-CCTTGCCGCCCAGGCGCACAGCG-3') to detect the transcript encoding the C-protein β -subunit-like (*CBLP*), used here as a loading control. Five independent transformants were tested in each case. For *ccs4* (vector), transformants are numbered from 1 to 5. For *ccs4* (*CCDA*↑), transformants #a, #a₁, #a₂ and transformants #b₁ and #b₂ show weak and strong suppression of the photosynthetic deficiency, respectively. Vector + *CCDA* corresponds to the pSL18-*CCDA* construct used to obtain the transformants #a, #a₁, #a₂ and #b₁. Transformant #b₂ was obtained by co-transformation of the pSL18 plasmid and the *CCDA* cDNA cloned (without promoter and terminator sequences) in a pBluescript vector. M for DNA marker. PCR amplification products were separated by electrophoresis in agarose gel and ethidium bromide stained. The gel was imaged using an imaging system.