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*, based on 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.
INTRODUCTION

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 sulphydryls in the apocytochrome $c$ (THONY-MEYER 1997). The cysteine sulphydryls 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 $c$ (HARTSHORNE et al. 2006; JUNGST et al. 1991; RIOS-VELAZQUEZ et al. 2001). 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 (BONNARD et al. 2010; FERGUSON et al. 2008; HAMEL et al. 2009; KRANZ et al. 2009; 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 reduction pathway. They are postulated to act sequentially to reduce the disulfide bonded CXXCH in apocytochrome $c$ prior to the heme ligation (ALLEN et al. 2003; KADOKURA et al. 2003; MAPLLER and HEDERSTEDT 2006; RITZ and BECKWITH 2001). 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 (reviewed in KADOKURA and BECKWITH 2010; MAPLLER and HEDERSTEDT 2006; MESSENS and COLLET 2006). Inactivation of the disulfide reduction pathway in bacteria results in a cytochrome $c$ deficient phenotype and it is believed that the
apocytochrome c CYCH 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 soluble cytochrome c6, the two c-type cytochromes required for photosynthesis (HOWE and MERCHANT 1992). In Chlamydomonas, cytochrome f and cytochrome c6 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 c6 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 b6 of the cytochrome b6f 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 b6 is dependent upon the CCB factors and takes place on the stromal side of the thylakoid membrane (KURAS et al. 1997; KURAS et al. 2007; LEZHNEVA et al. 2008; LYSKA et al. 2007; SAINT-MARCOUX et al. 2009).

The operation of a disulfide reducing pathway in the context of plastid cytochrome c assembly was first suspected based on 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 b6f assembly defect (PAGE et
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_{6}f assembly (Gabilly et al. 2010; Lennartz et al. 2001). The recombinant form of CCS5/HCF164 can reduce a disulfide at the CXXCH motif of apocytochrome f (Gabilly et al. 2010; Lennartz et al. 2001; Motohashi and Hisabori 2006).

In this paper, 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.

**MATERIAL 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 m′) and a cell-wall minus derivative ccs4 strain was identified. Wild type stains were CC124 and CC2677. Strains were grown at 22–25 °C in TAP liquid or solid medium (HARRIS 1989) with or without copper supplementation under dim light (25 μmol/m²/s) for ccs4 and ccs5 strains or under standard illumination for wild type strains (300 μmol/m²/s) as described in (HOWE and MERCHANT 1992). Copper deficient media are used to induce the expression of cytochrome c₆ (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 μmol/m²/s). An eight kb BamHI and a one kb SacII fragments containing the CCS4 gene were isolated from a complementing cosmid and cloned in pBluescript SK vector yielding the pSK-CCS4 BamHI and pSK-CCS4 SacII plasmids, respectively. The coding sequence of CCS4 (from ATG to stop) was cloned at EcoRI and XbaI sites of pSL18 (POLLOCK et al. 2004) between the PSAD promoter and terminator using Pccs4-ORF2-NdeI (5′-AACCCATATGTCGACTGGCATTGAGG-3′) and L-Pccs4-ORF2-XbaI (5′-AACTCTAGATCACTTGGTTGCCTGC-3′) as primers and pSK-CCS4 SacII 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 EcoRI and XbaI sites between the PSAD promoter and terminator of pSL18 via in-fusion technology (Clontech) using PORF5-F-EcoRI (5′-CGATAAGCTTGATATCGAATTCATGGCTATTTCAAAAGGCATTGAGG-3′) and PORF5-R-XbaI (5′-GGTCCAGCTGCTGCCATCTAGATCAGTCCTGCTGCTTGGCCTGCTCCTGG-3′) as primers and pSK-CCS4 SacII 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-NdeI (5’-GGGAATTCCATATGCGAACCGCCATGCATTTAG-3’) and ccdA-EcoRI (5’CGGAATTTCACGAGGGCACCAGGCGCG-3’) as NdeI and EcoRI engineered primers, respectively. The PCR product was cloned at the NdeI and EcoRI sites and yielded pSL18-CCDA.

RNA extraction and real time PCR: Wild type CC124, mutant strains *ccs4-F2D8* or *ccs4-F2-D8 arg7-8* transformed with the empty cosmid pCB412 or co-transformed with pCB412 and pSK-CCS4 *SacII (ccs4-Sac)* or with pCB412 and pSK-CCS4 *BamHI (ccs4-Bam)* were grown in TAP medium at 25°C with 25 μmol/m²/s of light. At about 6 x 10⁶ cells per 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’-GCTTCTCCCTGCAGCCGTCCT-3’ and 5’-GCAGGATCAAGCAGCGACAAGT-3’ for *CCS1*; 5’-TGGTTGCTGCTCAGGAGGAC-3’ and 5’-GCACGGCGAGCTCAGGATG-3’ for *CCS4*: 5’-GCCGGGTCGAGGTTATGG-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 LinRegPCR 11.x to obtain the mean PCR efficiency for each gene (RUIJTER *et al.* 2009). Transcript levels for the genes of interest (*gi*) were normalized to the transcript levels of the *CBLP* gene encoding the C-protein β-subunit-like. Relative transcript level (RTL) were
calculated as followed: \( \text{RTL} = 1000 \times [\text{mean PCR efficiency for } \text{CBLP}]^{C_{\text{CBLP}}} \times [\text{mean PCR efficiency for } gi]^{C_{gi}} \).

**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**

The **CCS4 gene product may participate in disulfide reduction:** Based on 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 (Bardischewsky and Friedrich 2001; Beckett *et al.* 2000; Deshmukh *et al.* 2000; Erlendsson and Hederstedt 2002; Feissner *et al.* 2005). Moreover, we have shown that *ccs5* could be rescued by DTT (Gabilly *et al.* 2010; Page *et al.* 2004). As shown in Figure 1A, addition of DTT to minimal medium can rescue the photosynthetic deficiency of *ccs4*. We noted that MESNA, 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 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 (Gabilly et al. 2010; Page et al. 2004), 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 one kb \( SacI \) fragment suggesting that the \( CCS4 \) gene is very small (Figure 2A). This one kb fragment restored photosynthetic growth (Figure 2A), fluorescence rise and decay kinetics, indicating that the cytochrome \( b_6f \) complex is functional (Figure 2B), and the accumulation of holoforms of cytochrome \( f \) and \( c_6 \) to wild type levels (Figure 2C).

**The \( CCS4 \) gene encodes a unique protein with no known motif:** RT-PCR experiments showed that the genomic region corresponding to the one kb \( SacI \) 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 6 fold 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 number 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 a 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). Based on the positive-inside rule that governs the topology of bacterial and thylakoid membrane proteins (Gavel et al. 1991; VonHeijne 1989), 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 one kb
SacII 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 non-sense mutation at residue Q50 in the predicted sequence (CAG to TAG) and presumably produces a non-functional 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 (PmR) cassette as a selectable marker. The resulting construct (pSL18/CCS4-ORF1) was introduced in the ccs4 mutant. Out of 22 PmR transformants, 12 were able to grow photosynthetically and displayed wild type fluorescence rise and decay kinetics (Figure 2AB). 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 one kb SacII genomic fragment, suggesting that ORF1 encodes the CCS4 gene product (Figure 2ABD).

In order 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. Out of 103 PmR transformants, 43 exhibited partial complementation of the photosynthetic growth defect and pseudo-wild type fluorescence rise and decay kinetics (Figure 2AB). 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 the one 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 PmR transformants tested (Figure 2A). 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 (Gabilly *et al.* 2010; Motohashi and Hisabori 2006; Motohashi and Hisabori 2010; Page *et al.* 2004). Real time PCR experiments showed no reduction in the abundance of the *CCDA* and *CCS5* transcripts in response to the *ccs4* mutation (Figure 3 and 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 only occur upon increased expression of the CCDA transcript. In order to test this, we cloned the CCDA cDNA (from ATG to stop) in front of the PSAD promoter, 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 selected on the basis of their resistance to paromomycin (PmR), a trait conferred by the selectable marker on the construct. Out of 44 PmR transformants, 14 were suppressed for the CCS phenotype based on the partial restoration of the photosynthetic growth defect (Figure 6A). While 12 transformants were weakly suppressed, two displayed an increased level of phototrophic growth (Figure 6A). To confirm our results, we co-transformed the ccs4 mutant with the PmR-cassette containing vector pSL18 and a plasmid containing only the full length CCDA cDNA (lacking promoter and terminator sequences for expression). Out of 45 PmR transformants, 15 were weakly suppressed for the photosynthetic defect while two 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. Out of 22 PmR transformants, 12 displayed photosynthetic growth and fluorescence transients indistinguishable
from wild type (Figure 6AB). No photosynthetic transformants were obtained among the 45 PmR transformants generated with the empty vector pSL18, ruling out the possibility that the two classes of suppressed transformants we recovered with pSL18/CCDA resulted from reversion to photosynthetic proficiency. The CCDA-dependent suppression correlated with partial restoration of the cytochrome $b_6f$ activity, and therefore 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 the weakly and strongly suppressed transformants (Figure S1, supplemental data).

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 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 paper, 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 (BONNARD et al. 2010; FERGUSON et al. 2008; HAMEL et al. 2009; KRANZ et al. 2009; SANDERS et al. 2010). The ability of exogenous thiol compounds to by-pass mutations inactivating the disulfide reducing components (BARDISCHEWSKY and FRIEDRICH 2001; BECKETT et al. 2000; DESHMUKH et al. 2000; ERLENDSSON and HEDERSTEDT 2002; FEISSNER et al. 2005; SAMBONGI and FERGUSON 1994) 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 suggest 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 (GABILLY et al. 2010; LENNARTZ et al. 2001; MOTOHASHI and HISABORI 2006). 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 (Gabilly et al. 2010; Motohashi and Hisabori 2006; Motohashi and Hisabori 2010; Page et al. 2004). Thioredoxin-\textit{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 \textit{Arabidopsis} thylakoids by recombinant spinach thioredoxin-\textit{m} (Motohashi and Hisabori 2006; Motohashi and Hisabori 2010).

\textit{Function of the CCS4 protein?}: It is unlikely that CCS4 has a reducing activity in the assembly process, based on the absence of motifs/residues in the protein sequence implying such an activity (Figure 4). One possibility is that the \textit{ccs4} mutation results in a loss of CCDA function. This is compatible with the fact that 1) \textit{ccs4} can be partially rescued by DTT (Figure 1), as seen in bacterial \textit{ccdA/dsbD} mutants that are restored for cytochrome \textit{c} assembly in the presence of exogenous thiols (Beckett et al. 2000; Deshmukh et al. 2000; Sambongi and Ferguson 1994) and 2) expression of \textit{CCDA} can partially by-pass the \textit{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 \textit{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 \textit{c} \textit{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 2 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/activity is influenced by the presence of single transmembrane proteins (PETERS et al. 2008; SCHULZ et al. 1999; YU et al. 1999). 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 (AHUJA and THÖNY-MEYER 2005; GOLDMAN et al. 1997; RICHARD-FOGAL et al. 2008; SCHULZ et al. 2000). CcmD was shown to physically interact with the heme relay Ccm components and also influence their stability in the membrane (AHUJA and THÖNY-MEYER 2005; RICHARD-FOGAL et al. 2008; SCHULZ et al. 2000). 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). Beside Volvox carteri and Dunaliella salina, we could not find any CCS4 orthologs in other genomes, including genomes of green algae such as Ostreococcus and Chlorella. One possibility is that the function of CCS4 is dependent upon the overall charge of the protein rather than 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).

REFERENCES


ALLEN, M. D., J. A. DEL CAMPO, J. KROPAT and S. S. MERCHANT, 2007 FEAT1, FEAT2, and FRE1, encoding two homologous secreted proteins and a candidate ferrireductase, are expressed coordinately with FOX1 and FTR1 in iron-deficient Chlamydomonas reinhardtii. Eukaryot. Cell 6: 1841-1852.


HARTSHORNE, S., D. J. RICHARDSON and J. SIMON, 2006 Multiple haem lyase genes indicate substrate specificity in cytochrome c biogenesis. Biochem. Soc. Trans. 34: 146-149.


MOTOHASHI, K., and T. HISABORI, 2010 CcdA is a thylakoid membrane protein required for the transfer of reducing equivalents from stroma to thylakoid lumen in the higher plant chloroplast. Antioxid. Redox Signal. 13: 1169-1176.


SCHULZ, H., R. A. FABIANEK, E. C. PELLICIOLI, H. HENNECKE and L. THÖNY-MEYER, 1999 Heme transfer to the heme chaperone CcmE during cytochrome c maturation requires the CcmC protein, which may function independently of the ABC-transporter CcmAB. Proc. Natl. Acad. Sci. USA **96**: 6462-6467.


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**FIGURE LEGEND**

**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, Minimal medium with or without 2 mM DTT. Cells grown heterotrophically were incubated at 25°C for 7 days with 20 µmol/m²/s of light. Cells grown phototrophically with or without DTT were incubated at 25°C for 14 days with 250 µmol/m²/s of light. Cells grown phototrophically showed the best rescue with 2 mM DTT.

**B.** Fluorescence kinetics indicate a partial restoration of cytochrome b₆f in DTT-treated ccs4 cells.

The fluorescence induction and decay kinetics observed in a dark to light transition of ccs4 grown in absence or presence of 1 mM and 2 mM DTT are shown compared to those of wild type (WT). Fluorescence transients were measured using Handy Fluorcam from Photon System Instruments. The fluorescence is in arbitrary units (A.U.) and recorded over a 3s illumination period. The rise and plateau curve for ccs4 is a signature of a specific block in electron transfer at the level of cytochrome b₆f 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 cytochrome b₆f, 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 b₆f 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 (Acetate, low light) in absence or presence of 1 or 2 mM DTT. Samples of wild type (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 CF1 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

ABD.: The ccs4-F2D8 arg7-8 strain was transformed with pSL18 (ccs4), pSL18 carrying a one kb genomic fragment with the CCS4 gene (CCS4), pSL18 expressing the full length CCS4 coding sequence (CCS4, ORF1) or pSL18 expressing a truncated form of the CCS4 protein (CCS4, ORF2). C.: The ccs4-F2D8 arg7-8 strain was transformed with pCB412 (ccs4) or co-transformed with pCB412 and pSK-CCS4-SaclI carrying a one kb genomic fragment with the CCS4 gene (ccs4 (CCS4)). Only one representative transformant is shown in ABC. In D., two representative transformants (CCS4, ORF2) are shown. In BCD., 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 (heterotrophic conditions, 20 µmol/m²/s of light) and Minimal medium (phototrophic conditions, 250 µmol/m²/s of light) and incubated at 25°C for 1 week and 3 weeks, respectively.
**B. Fluorescence kinetics indicate restoration of cytochrome b$_{6}$f 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 3s 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 c$_{6}$ 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 CF$_{1}$ that serves as loading control. Samples corresponding to 16 µg of chlorophyll were separated in 15% native acrylamide gel to detect cytochrome c$_{6}$. 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 c$_{6}$ and CF$_{1}$.

**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.

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**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 co-transformed by pCB412 and pSK-CCS4 *BamHI* (#4) or co-transformed by pCB412 and pSK-CCS4 *SacII* (#5). Relative transcript levels (RTL) represent the mean levels of three independent experiments, each analyzed in technical triplicates. RTL values are relative to the *CBLP* levels and were calculated as described in the materials and methods section. The abundance of *UBQ2* is shown as a control.

**Figure 4. Alignment of Chlamydomonas, Volvox and Dunaliella CCS4 proteins**

Sequences of *Chlamydomonas reinhardtii* (Crein, accession number:ADL27744), *Volvox carteri* (Vcart, accession number:FD920844.1), *Dunaliella salina* (Dsali1, accession number:BM447122.1 and Dsali2, accession number: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 shaded in black. The putative membrane anchor is boxed. The black downward arrowhead indicates the position of the methionine in the truncated CCS4 form and the grey downward arrowhead indicates the Q residue that is mutated to a stop codon in the *ccs4-F2D8* strain.

**Figure 5. Accumulation of the CCS5 protein in ccs4.**

Total protein (corresponding to 20 μg 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) were analyzed by SDS–PAGE (12%) and immunoblotting with antiserum 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.
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), pSL18 expressing the CCDA ORF (CCDA↑, transformants #a and #b₁) or co-transformed 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 µmol/m²/s of light) and minimal medium (phototrophic conditions, 300 µmol/m²/s of light) and incubated at 25°C for 1 week and 3 weeks, respectively.

B. Fluorescence kinetics indicate partial restoration of cytochrome b₅f 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.
Figure 4

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**Sequence Alignment**

**Figure 4**

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Figure 5
Figure 6

A

B

C

Fluorescence (A.U.)

Time (s)

Cyt f heme stain

anti-Cyt f

anti-CF₁