A Disulfide-Reducing Pathway Required For Plastid Cytochrome *c* Assembly

DISSERTATION

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By

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ABSTRACT

The formation and reduction of disulfide bonds is essential for the folding, stability and function of a large number of proteins. While the Dsb pathway ("Dsb" for Disulfide bond) controls thiol/disulfide chemistry in the bacterial periplasm, the question of how this process is performed in the thylakoid lumen, the topologically equivalent compartment in plastids and in cyanobacteria, has yet to be explored. The occurrence of disulfide-bonded proteins and the requirement for reduced cysteines in the assembly of cytochromes *c* in this compartment suggests that thiol/disulfide chemistry is also enzymatically assisted. From our study, using the unicellular green alga, *Chlamydomonas reinhardtii* as model organism, we have identified three novel components CCS4, CCS5 and CCDA operating in a trans-thylakoid disulfide-reducing pathway, which is required for the assembly of cytochromes *c*, a class of metalloproteins with a covalently attached heme. The *ccs4* and *ccs5* mutants are photosynthetic deficient and exhibit a block in the assembly of cytochromes *f* and c_6, two *c*-type cytochromes of the algal photosynthetic apparatus, involved in electron transfer. CCS5 is a membrane anchored lumen-facing thioredoxin-like protein that we show is the functional homolog of *Arabidopsis thaliana* HCF164, previously identified as being involved in photosynthesis. A disulfide-reducing activity for CCS5 is inferred from the findings that *ccs5* is chemically rescued by exogenous thiols and that recombinant CCS5 reduces a disulfide in the C*XX*CH heme

binding motif of apocytochrome *c*. CCS4 is a small protein with a hydrophobic amino-terminus, a hydrophilic carboxyl-terminal domain containing several charged residues and no noticeable motif suggestive of a biochemical activity. Its involvement in a disulfide-reducing pathway is based on the thiol-dependent photosynthetic rescue of the *ccs4* mutant and also on the ectopic expression of plastid CCDA, an ortholog of the bacterial thiol-disulfide transporter. Because heme is covalently linked to the heme binding motif, we propose that CCS5 controls plastid cytochrome *c* assembly by maintaining the C*XX*CH cysteines under a reduced form prior to the heme attachment in the thylakoid lumen. We postulate that CCS5 is maintained in a reduced state by the thylakoid membrane transporter CCDA whose activity/stability is regulated by CCS4 via a yet-to-be defined mechanism.

To my father

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FIELDS OF STUDY

Major Field: Molecular, Cellular and Developmental Biology

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INTRODUCTION

1.1. Disulfide bond formation

1.1.1. A post-translational modification for protein folding, stability and function

In all living organisms, proteins are synthesized as linear polypeptides followed by rapid folding into their unique three-dimensional structures. Proper folding is a necessary step for the biological activity of all proteins. Disulfide bonds are one of the few post-translational covalent modifications that occur during protein folding. Failure to form the correct disulfide bonds is likely to cause protein aggregation and degradation by cellular proteases (DEPUYDT *et al.* 2011; MAMATHAMBIKA and BARDWELL 2008; NAKAMOTO and BARDWELL 2004). The stability of a protein in its final folded structure may be governed by cysteine-cysteine linkage. The folding process for such protein will be faster, as the protein can take on many fewer conformations than a protein whose cysteines remain free. A protein with disulfide bonds is also more stable thermo-dynamically (NAKAMOTO and BARDWELL 2004; ORTENBERG and BECKWITH 2003). The function of a protein may be governed by the oxidation-reduction cycling of a disulfide bond (ORTENBERG and BECKWITH 2003). Disulfides can be classified into catalytic disulfides and allosteric

disulfides. Catalytic disulfides are typically found at the active site of enzymes such as oxidoreductases. Dithiols/disulfides are transferred via their very reactive cysteines to a protein substrate and result in the formation, reduction or isomerization of disulfide bonds in the substrate. Allosteric disulfides regulate function in a nonenzymatic way by mediating changes in the protein structure (HOGG 2003; SCHMIDT *et al.* 2006). Hence, disulfide bond formation in proteins is required not only for folding but also for stability and function.

The formation of disulfide bonds is thermodynamically coupled to the process of protein folding. The folded conformation stabilizes the disulfide bond to the same extent that the conformation is stabilized by the formation of that particular disulfide bond. With entropy as a measure of flexibility, disulfide bonds are involved in protein stability by restricting the flexibility of the denatured state therefore by lowering the entropy of the denatured state compared to the native state. Free energy is a measure of stability which is increased for a less flexible protein. From these features, the contribution of a disulfide bonded active site to the stability of a protein is limited compared to structural disulfides (MATSUMURA *et al.* 1989).

1.1.2. Disulfide bond chemistry

The chemistry of disulfide bond formation and breakage are well established. Formation of a protein disulfide bond is an oxidation reaction, in which two electrons are removed from the protein. In contrast breakage of the protein disulfide bond is a reduction

reaction, in which two electrons are donated to the protein (ITO and INABA 2008; KADOKURA and BECKWITH 2010).

The formation of a disulfide bond from two thiols also requires the presence an oxidant or electron acceptor. *In vivo*, molecular oxygen is commonly used as the electron acceptor (SEVIER and KAISER 2002). Disulfide bonds can be formed spontaneously, *in vitro*, in the presence of molecular oxygen. However, this type of spontaneous, random air-oxidation reaction is very slow and cannot account for the rapid rates of disulfide bond formation needed by the cell (MAMATHAMBIKA and BARDWELL 2008).

1.1.3. A process enzymatically assisted, *in vivo*

Disulfide bond formation is a catalyzed process *in vivo* (NAKAMOTO and BARDWELL 2004). Genetic studies in bacteria revealed that enzymatic systems in extracytoplasmic compartments are necessary for the efficient formation of protein disulfide bonds. In the absence of such a system, the formation of a disulfide bond is extremely slow. The absence of disulfide bonded proteins in the cytoplasm cannot only be attributed to the generally reducing environment of this compartment. On the other hand, proteins containing stable structural disulfide bonds are usually exclusively found in highly oxidizing cellular compartments (e.g. bacterial periplasm) (FABIANEK *et al.* 2000; KADOKURA *et al.* 2003; ORTENBERG and BECKWITH 2003; RITZ and BECKWITH 2001). The high potential for forming disulfide bonds is not simply due to the presence of oxygen; these compartments contain enzymatic systems that catalyze the formation of disulfide bonds.

Important progress has been made towards understanding how disulfide bonds are formed in cellular proteins. It is now clear that the prokaryotic and eukaryotic pathways of disulfide bond formation have remarkable similarities, which include their orchestration at the mechanistic level. Enzymes that are capable of catalyzing protein disulfide bond formation are members of a large collection of thiol-disulfide oxidoreductases found in all living cells. Most of these enzymes belong to the thioredoxin superfamily defined by an active site containing a C*XX*C motif (cysteines separated by two amino acids) and a thioredoxin fold similar to the three dimensional structure of the prototypical thioredoxin 1 of *E.coli*. Thioredoxins are ubiquitous small soluble proteins with redox-active cysteines, conserved in a C*XX*C motif, that catalyses thiol-disulfide exchange reactions (FABIANEK *et al.* 2000; KADOKURA *et al.* 2003; ORTENBERG and BECKWITH 2003; SEVIER and KAISER 2002).

1.1.4. Mechanism of thiol-disulfide exchange

In vivo, the most common mechanism for the formation of protein disulfide bonds is a thiol-disulfide exchange reaction of free thiols with a disulfide-bonded species. A thiol-disulfide exchange reaction can occur between a disulfide-bonded protein and any sulfhydryl-containing substrate, including small thiol-containing compounds, such as glutathione (MAMATHAMBIKA and BARDWELL 2008; SEVIER and KAISER 2002). Thiol-disulfide exchange reactions determine mechanistically how disulfide bond formation occurs in all living organisms (see Figure 1.1).

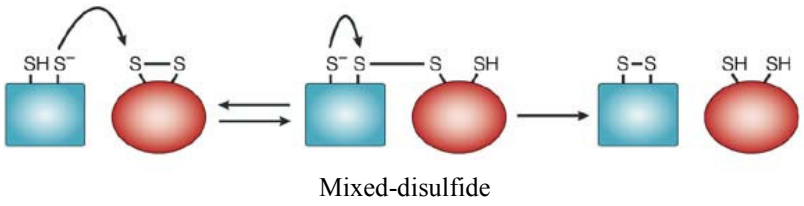


Figure 1.1. Thiol-disulfide exchange reactions between proteins.

Disulfide bonds in proteins are formed by two thiol-disulfide exchange reactions. In a first exchange reaction, a thiolate anion ($-S^-$), which is formed by the deprotonation of a free thiol, displaces one sulfur of the disulfide bond in the oxidized species. This results in the formation of a transient mixed-disulfide bond between the two proteins, or between a protein and redox molecule such as glutathione. In a second exchange reaction, the remaining thiolate anion attacks the mixed-disulfide bond and resolves it. The net result of this thiol-disulfide exchange process is the oxidation of the originally reduced protein, and the concomitant reduction of the initially oxidized redox species. Such exchange reactions can also occur intramolecularly, leading to the rearrangement of disulphide bonds in a single protein. Source: (SEVIER and KAISER 2002).

1.2. Disulfide bond formation in bacteria and mitochondria

1.2.1. Disulfide bond formation in Gram-negative bacteria

The pathways of disulfide bond formation have been best characterized in *Escherichia coli*, a Gram-negative bacterium. Disulfide bond formation takes place in the periplasm, a compartment that separates the bacterial outer membrane from the inner membrane and significantly more oxidizing than the cytoplasm (see Figure 1.2) (DEPUYDT *et al.* 2011).

In *E.coli*, several hundred proteins containing multiple cysteines are exported to the periplasm (DEPUYDT *et al.* 2011; DUTTON *et al.* 2008; HINIKER and BARDWELL 2004; MCCARTHY *et al.* 2000). These proteins are potential substrates for disulfide bond forming enzymes.

Disulfide bonds are introduced by the Dsb proteins ("Dsb" for Disulfide bond). The Dsb pathway includes DsbA and DsbB, which are involved in disulfide bond formation, and DsbC and DsbD, which are involved in disulfide bond isomerization (see Figure 1.2) (DEPUYDT *et al.* 2011; FABIANEK *et al.* 2000; HERRMANN *et al.* 2009; INABA 2009; ITO and INABA 2008; KADOKURA and BECKWITH 2010; KADOKURA *et al.* 2003; NAKAMOTO and BARDWELL 2004; RITZ and BECKWITH 2001).

Despite a large number of potential substrates, the Dsb proteins involved in disulfide-bond formation are not essential for viability although mutants exhibit pleiotropic phenotypes (HERRMANN *et al.* 2009; KADOKURA *et al.* 2003). *DsbA* mutants show hyper sensitivivity to the reductant dithiothreitol (DTT), heavy metal ions, loss of motility and poor growth in minimal media (BARDWELL *et al.* 1991; KADOKURA *et al.* 2003; MISSIAKAS *et al.* 1993). Surprisingly, *dsbA* mutants are particularly healthy when grown on rich media. This is in part may be due to the presence of small oxidant molecules in rich media (BARDWELL *et al.* 1993). The sensitivity of *dsbA* mutants to DTT can be explained by the fact that, in absence of DsbA, a fraction of essential periplasmic proteins requiring disulfide bond(s) to be functionally active are no longer oxidized by these non-specific oxidants. The DTT would somehow interfere with the oxidizing ability of the small molecules, preventing the necessary oxidation of essential proteins in *dsbA* mutants. For the sensitivity to heavy metal ions, it is believed that mercury and cadmium have high affinity for thiol groups of proteins. Target proteins of DsbA remain reduced in *dsbA* mutants. The thiols are free to react with mercury and cadmium resulting in a toxic increased concentration of heavy metal ions inside the cell (STAFFORD *et al.* 1999).

FlgI is a flagellar motor protein functional in the oxidized state. Therefore, if the protein is not disulfide bonded, the bacteria are nonmotile (DAILEY and BERG 1993). The loss of motility can be visualized on low concentration agar. Alkaline phosphatase from *E. coli* is a homodimeric protein found in the periplasmic space. Each subunit contains two intramolecular disulfide bonds required for its activity (SONE *et al.* 1997). The motility test and the measure of the alkaline phosphatase activity are frequently used to assay for defects in disulfide bond formation. In addition, *dsbA* mutants in many pathogenic bacteria are avirulent since virulence components such as pili and toxins contain disulfide bonds (PEEK and TAYLOR 1992). In *dsbA* or *dsbB* mutants, many proteins such as alkaline phosphatase, β-lactamase, the outer membrane protein OmpA or FlgI are rapidly degraded due to the absence of disulfide bonds necessary for their stability (BARDWELL *et al.* 1991; DAILEY and BERG 1993).

1.2.1.1. DsbA catalyzes disulfide bond formation

DsbA is a 21 kDa soluble thiol-disulfide oxidoreductase involved in the disulfide bond formation of newly translocated proteins in the periplasm. The oxidation of reduced, unfolded proteins is random and extremely rapid via a disulfide exchange reaction (JOLY and SWARTZ 1997; KISHIGAMI *et al.* 1995; WUNDERLICH *et al.* 1995; WUNDERLICH *et al.* 1993; ZAPUN and CREIGHTON 1994). Like most thiol-disulfide oxidoreductases, DsbA has a thioredoxin (Trx)-fold and carries a C*XX*C catalytic motif (CPHC in *E.coli* DsbA) (ATKINSON and BABBITT 2009; COLLET and MESSENS; MARTIN *et al.* 1993). DsbA is a powerful oxidant, one of the most oxidizing proteins known. *E.coli* DsbA has a redox

potential of -119 mV (ASLUND *et al.* 1997; ZAPUN *et al.* 1993), but DsbA homologs from other bacteria such as *Neissaria meningitides* (LAFAYE *et al.* 2009; VIVIAN *et al.* 2009) and *Pseudomonas aeruginosa* (SHOULDICE *et al.*) are even more oxidizing with a redox potential of -80 and -95 mV, respectively. For comparison, thioredoxin has a much lower standard redox potential of -270 mV. The equilibrium redox potential is a quantitative measure of a protein's propensity to be in either a reduced or an oxidized state. It is determined experimentally by measuring the relative amounts of oxidized and reduced protein species in redox equilibrium with a compound of known redox potential. Glutathione, reduced or oxidized is commonly used (SEVIER and KAISER 2002).

DsbA has the ability to quickly introduce disulfide bond into its substrates, *in vivo* and *in vitro*. The origin of the extraordinary oxidizing power of DsbA comes from its intrinsic structural features. DsbA catalytic cysteine residues, Cys30 and Cys33 are conserved in a CPHC motif, located at the N-terminus of the first α-helix of the thioredoxin fold. The Cys30 has a very low pKa (3.5) compared to the pKa of about 8.7 for a normal cysteine. The low pKa of this first cysteine renders the disulfide bond of DsbA very unstable and therefore, very reactive, with a strong tendency to interact with reduced cysteines in substrate proteins. Such a low pKa is critical for the high oxidizing capacity of DsbA.

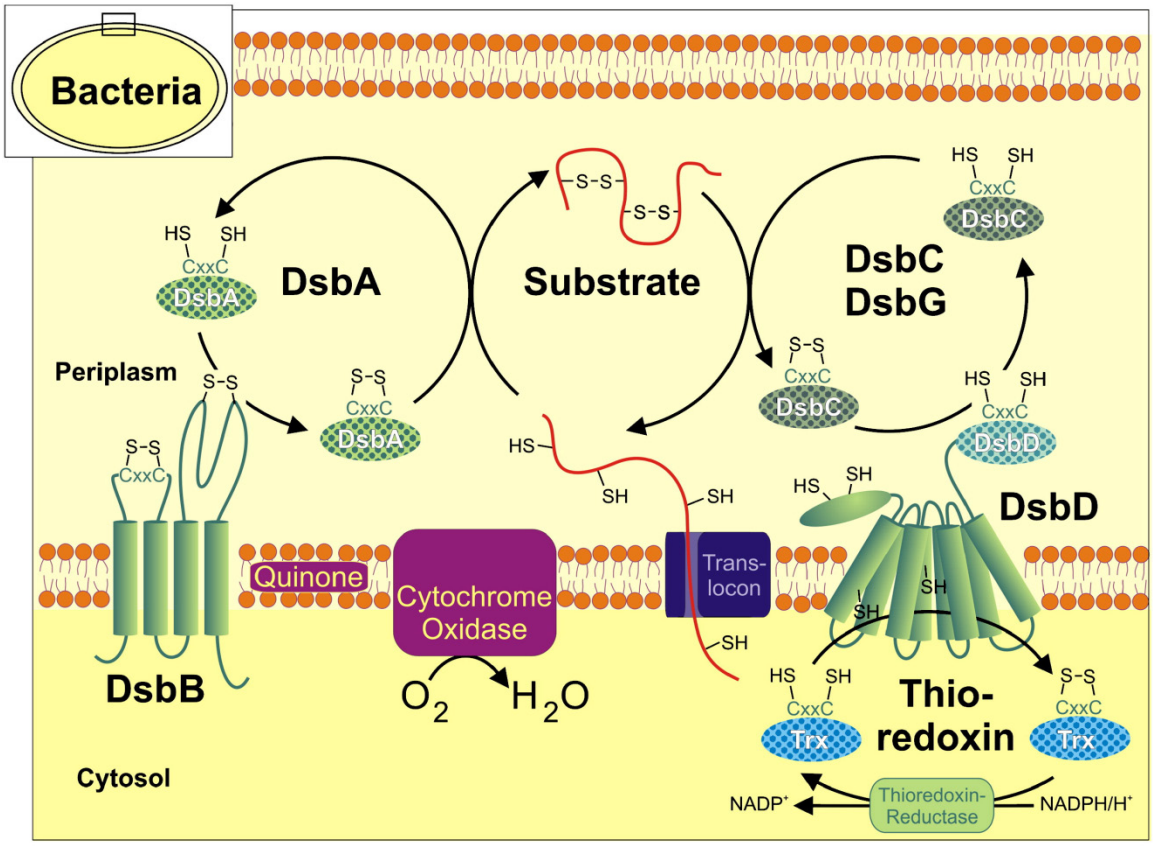


Figure 1.2. Protein oxidation in bacteria.

Proteins are secreted into the periplasm through the protein-conducting channel of the translocon. Either concomitant with or directly after protein translocation into the periplasm, thiol groups are oxidized by the soluble protein DsbA. DsbA is reoxidized by DsbB, a membrane protein that can transfer electrons further on *via* quinones and terminal oxidases to oxygen leading to the production of water. DsbC and DsbG counteract protein oxidation and are critical for the isomerization and folding of periplasmic proteins. These soluble proteins interact with the membrane protein DsbD which acts as a membrane transporter for thiol disulfide connecting DsbC and DsbG to the cytosolic thioredoxin reductase system. Thioredoxin domains are depicted as dotted ovals. Source: (HERRMANN *et al.* 2009).

The Cys30 residue allows the partial positive charge from the dipole of the α-helix to stabilize the thiolate (GUDDAT *et al.* 1997). Then, His32, which lies between Cys30 and Cys33, is hydrogen-bonded to Cys30 in the reduced but not in the oxidized DsbA,

providing additional stabilization of the thiolate anion of Cys30 (GUDDAT *et al.* 1997). Moreover, the structure of reduced DsbA reveals a number of potential hydrogen bonds that stabilize the thiolate anion of Cys30. One of them is a hydrogen bond between the thiolate anion of Cys30 and the thiol of Cys33. The other two hydrogen-bond interactions are between the thiolate anion of Cys30 and the backbone amide of His32 and Cys33 (GUDDAT *et al.* 1997). The difference in the stabilities of oxidized and reduced forms provides a thermodynamic driving force for substrate oxidation by DsbA.

The surface of DsbA's oxidized form is more flexible than its reduced form (VINCI *et al.* 2002). The greater flexibility of the oxidized form may facilitate the accommodation of various substrates while the more rigid nature of the reduced form may expedite the release of oxidized substrates. The surface structure of DsbA has a relatively deep hydrophobic groove running along side the CPHC active site. This groove has been postulated to be the binding site of the unfolded substrate but this hypothesis awaits experimental confirmation (GUDDAT *et al.* 1997).

The proposed model for disulfide bond formation by DsbA is a two step thiol-disulfide exchange reaction (KADOKURA and BECKWITH 2009). In the first step, a deprotonated cysteine of a substrate attacks the sulfur atom of Cys30 in the Cys30-Cys33 disulfide bond. This reaction generates an intermolecular disulfide bond between the cysteine of the substrate and Cys30 of DsbA and the reduction of Cys33 of DsbA. In the second step, another cysteine of the substrate is deprotonated and attacks the sulfur atom of the substrate cysteine that is disulfide bonded with Cys30 of DsbA. This reaction generates a stable disulfide bond in the substrate and DsbA becomes reduced. After transfer of its

disulfide bond to a target protein, DsbA must be reoxidized to repeat another catalytic cycle.

1.2.1.2. DsbB: a membrane protein that maintains DsbA in the oxidized active state

DsbB is a 20 kDa integral membrane protein that reoxidizes DsbA (BARDWELL *et al.* 1993). In wild type cells, DsbA is maintained exclusively in the oxidized state. In *dsbB-null* mutants, DsbA failed to be reoxidized and remained in the reduced state. *DsbB* mutants exhibit pleiotropic phenotypes such as hypersensitivivity to DTT and deficiency in disulfide bond formation for OmpA and β-lactamase, two periplasmic proteins (MISSIAKAS *et al.* 1993). These phenotypes resemble the phenotype of bacteria carrying a null mutation in *dsbA* gene as well as the *dsbA dsbB* mutant (MISSIAKAS *et al.* 1993).

Topological analysis of DsbB reveals four transmembrane helices. One pair of cysteines is located in each of the two periplasmic loops of the protein. The first pair of cysteines (Cys41 and Cys44) is in the N-terminal loop, the second (Cys104 and Cys130) is in the C-terminal loop. These cysteines can undergo oxidation and reduction cycles and are essential for the DsbB activity (JANDER *et al.* 1994). Like DsbA, DsbB contains a pair of cysteines conserved in a C*XX*C motif (Cys41 - Cys44) but unlike DsbA, the region surrounding the C*XX*C motif of DsbB does not contain a thioredoxin-like fold.

The fact that heme or quinone depleted cells accumulate reduced DsbA and DsbB suggests that the oxidizing power for protein disulfide bond formation, in *E.coli*, is provided by the respiratory chain (KOBAYASHI *et al.* 1997). Indeed, DsbB restores the

disulfide bond to DsbA by using the oxidizing power of the electron transport chain (BADER *et al.* 1999; KOBAYASHI *et al.* 1997).

DsbB generates a disulfide in DsbA by reducing quinones. Aerobically, the quinone is a ubiquinone. The reduced ubiquinone is then reoxidized by terminal cytochrome oxidases. Electrons are then transferred to molecular oxygen and give rise to the production of water (see Figure 1.2). Anaerobically, electrons are transferred from DsbB to menaquinone. The reduced menaquinone is then reoxidized by an anerobic electron-transport system. The ability of DsbB to use either ubiquinone or menaquinone as electron acceptors allows the DsbA-DsbB system to function under aerobic and anaerobic conditions (BADER *et al.* 1999).

The efficient oxidation of the DsbA-DsbB system lets believe that the cysteine residues in secreted proteins are converted to disulfide bonds already during their translocation across the inner membrane. This raises the question of how disulfide bond formation and protein secretion are coordinated. Proteins targeted to the periplasm are synthesized in the cytoplasm and possess a N-terminal export signal recognizable by an export machinery, either the TAT pathway or the Sec pathway (NATALE *et al.* 2008). Most of these proteins are transported unfolded across the inner membrane by the SecYEG translocon (in opposition to the TAT pathway where folded proteins are transported across the membrane). Depending on the properties of the signal sequence, proteins are secreted co-translationaly or post-translationaly (HUBER *et al.* 2005; JOSEFSSON and RANDALL 1983).

How disulfide bond formation is coordinated with the folding process of a protein in the periplasm is far from being understood. Kadura and Beckwith designed a *dsbA* mutant

forming stable complexes with alkaline phosphatase (PhoA) (KADOKURA and BECKWITH 2009), a periplasmic protein with two consecutive disulfide bonds (KAMITANI *et al.* 1992). From their studies, they concluded that disulfide bonds are preferentially introduced in a vectorial manner, from the N terminus to the C terminus, into PhoA molecules entering the periplasm. In addition, by fusing PhoA to different signal sequences, they were also able to study the influence of the mode of secretion (co- or post-translational) on the oxidative folding process. The co-translational export of proteins seems to favor the vectorial oxidation of the cysteine residues more than the post-translational export (KADOKURA and BECKWITH 2009). One possible explanation is that the rate of translocation is limited by the rate of protein synthesis when a protein is exported co-translationally. The polypeptide appears therefore more slowly in the periplasm, which favors the vectorial oxidation of the cysteine residues of the secreted polypeptide.

1.2.1.3. DsbC: a periplasmic Disulfide-Bond Isomerase

DsbA is a very strong oxidant and oxidizes thiol compounds extremely rapidly but none specifically. In any proteins with more than two cysteines, the potential for incorrect disulfide bond formation is high. Indeed, for example, a protein with four pairs of cyteines has less than 1% chance of acquiring the correct four disulfides by random oxidation. Therefore, DsbA has the potential of introducing non-native disulfides into proteins with multiple cysteines (RIETSCH *et al.* 1996). *In vivo*, DsbA is present in the oxidized state and has a very low ability to reduce incorrect disulfides. DsbA doesn't

have proofreading activity for improperly formed disulfides. Incorrect disulfides will need to be reshuffled and corrected before the protein can reach its proper structural confirmation. Disulfide reshuffling involves an intra molecular thiol/disulfide exchange reaction. In the periplasm, this reaction is catalyzed by the disulfide bond isomerase, DsbC (MISSIAKAS *et al.* 1994; RIETSCH *et al.* 1996).

In *E.coli* mutants lacking DsbC, some disulfide bonded proteins either have a reduced activity or are degraded. These proteins are periplasmic endoribonuclease RNAse I, periplasmic acid phosphatase AppA and periplasmic endonuclease -1 End1 (BERKMEN *et al.* 2005; HINIKER and BARDWELL 2004; MESSENS *et al.* 2007; VERTOMMEN *et al.* 2008).

A common feature of these proteins is that they all contain several disulfide bonds, one of which is at least non-consecutive. This feature favors the possibility of incorrect disulfide bond formation for these proteins resulting in their misfolding. As a consequence, they become inactive or degraded, in absence of an isomerase to correct the non-native disulfide bonds.

E.coli dsbC mutants also show an increased sensitivity to copper. In the presence of molecular oxygen, copper is a strong oxidant responsible for the misoxidation of proteins. The current explanation is that the copper sensitivity of *E.coli dsbC* mutants is due to the inability of the mutants to repair the Cu-induced misoxidized proteins of the periplasm (HINIKER *et al.* 2005).

DsbC is a soluble, homodimeric molecule with a V-shaped structure. Each monomer of 23 kDa consists of a C-terminal thioredoxin domain with a C*XX*C active site and an N-terminal dimerization domain connected by a linker (MCCARTHY *et al.* 2000). The two

cysteines (Cys98 and Cys101) in the active site of each monomer face the inside of the V-shape and are maintained reduced in the periplasm, in contrast to the C*XX*C motif of DsbA (JOLY and SWARTZ 1997). Although, the *in vivo* redox states of the catalytic cysteines of DsbA and DsbC are different, the redox potential of DsbA (-120mV) is similar to the redox potential of DsbC (-130mV) (ZAPUN *et al.* 1995). DsbC is kept reduced in the periplasm to enable the correction of non-native disulfides.

How does DsbC repair incorrect disulfide bonds in proteins? Two models have been proposed (Figure 1.3) (KADOKURA *et al.* 2003; RIETSCH *et al.* 1997). In both models, the reaction starts with the nucleophilic attack by Cys98 of DsbC on the incorrect disulfide bond resulting in a mixed-disulfide bond between DsbC and its substrate. The mixed-disulfide can be resolved in two ways. In the first model, the mixed-disulfide is attacked by a third cysteine of the substrate, resolving the complex and allowing a more stable disulfide bond to form in the substrate. DsbC is back to the reduced form. In this case, DsbC is acting as a true protein disulfide isomerase (PDI). In the second model, the mixed-disulfide is attacked by Cys101 of DsbC, generating a reduced substrate and an oxidized DsbC. Next, the reduced substrate will be reoxidized randomly by DsbA for a new chance to form the correct disulfide bond. In this model, DsbC is acting as a disulfide bond reductase rather than as an isomerase. In both models, the active site of DsbC must be in the reduced state in order for the enzyme to carry out the first step in the repair of non-native disulfide bonds. Theoretically, non-native disulfide bonds can be repaired by repeating the isomerization or reduction-oxidation reaction until the substrate protein has reached its native 3D structure. In addition to the isomerase or reductase

activity, DsbC has a chaperone activity. DsbC can bind unfolded proteins. The binding activity does not require the active-site cyteine residues (KADOKURA and BECKWITH 2010).

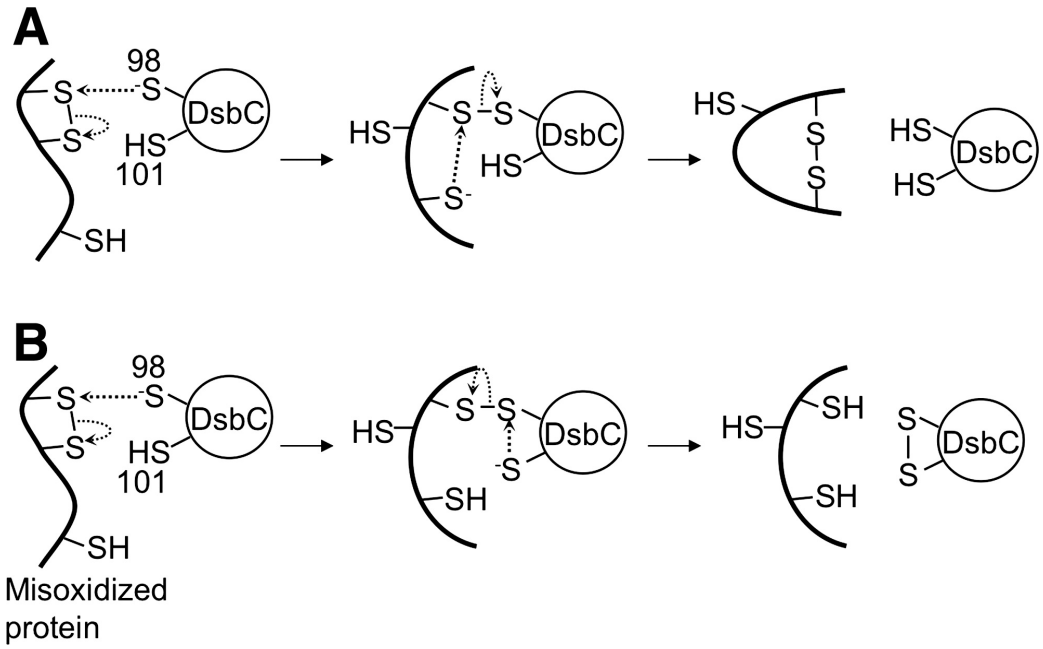


Figure 1.3. Disulfide-bond isomerization (A) and reduction (B) by DsbC.

Two mechanisms have been proposed for the repair of a misoxidized cysteine pair by DsbC. After the reduction of the substrate protein in sequence (**B**), DsbA can reoxidize the substrate, potentially generating the correct disulfide bond (not shown). Source: (KADOKURA and BECKWITH 2010).

1.2.1.4. DsbG: a periplasmic thiol-oxidoreductase, homolog of DsbC, protects single cysteines from hyperoxidation

A few years after the discovery of DsbC, a periplasmic thiol-oxidoreductase, DsbG, which is an ortholog of the disulfide-bond isomerase was identified (ANDERSEN *et al.* 1997; BESSETTE *et al.* 1999). DsbG shares 24% amino acid sequence identity with DsbC. The size of the monomer is 26 kDa. Like DsbC, DsbG is homodimeric and forms a V-

shaped structure with its N-terminal dimerization domain and its C-terminal thioredoxin domain connected by a linker helix (HERAS *et al.* 2004). The two cysteines (Cys109 and Cys112) of the C*XX*C motif active-site are maintained reduced *in vivo* (BESSETTE *et al.* 1999). DsbG has a redox potential of -126 mV, similar to that of DsbC (BESSETTE *et al.* 1999). DsbG and DsbC also have similar structure although significant differences do exist (HERAS *et al.* 2004; MCCARTHY *et al.* 2000). Most strikingly, the size of the cleft formed by the V-shaped structure of DsbG is twice the size of that in DsbC. DsbG also has several acidic residues lining the cleft which form a negatively charged surface not present in DsbC. From these properties, it has been proposed that DsbG can interact with larger proteins and needs less hydrophobic surfaces compared to the substrates of DsbC. DsbG exhibits molecular chaperone and protein disulfide isomerase activities *in vitro* (BESSETTE *et al.* 1999; SHAO *et al.* 2000). However, its protein disulfide isomerase activity is much weaker compared to the one of DsbC based on *in vitro* activities (BESSETTE *et al.* 1999; HINIKER *et al.* 2007). Consistent with these results, overexpression of DsbG suppresses some of the defects of *dsbC*-null mutants (BERKMEN *et al.* 2005; BESSETTE *et al.* 1999). Yet, the cellular function of DsbG remained unknown until recently. Depuydt *et al*. (DEPUYDT *et al.* 2009) have found that DsbG preferentially interacts with three periplasmic proteins, all belonging to the same family of the $_{L,D}$-transpeptidases and responsible for the cross linking of the major outer-membrane lipoprotein to the peptidoglycan of *E.coli* (MAGNET *et al.* 2007). One important feature about these proteins is that they possess a sole cysteine essential for activity. Depuydt *et al*. showed that such a cysteine can be oxidized to a sulfenic acid in the periplasm and

that both DsbG and DsbC can protect the cysteine from sulfenylation. However, DsbG appears to be much more proficient than DsbC in protecting free cysteines in proteins from sulfenylation (DEPUYDT *et al.* 2009).

As mentioned earlier, the cleft formed by the V-shape of DsbG is much larger and more hydrophobic than the cleft of DsbC. This characteristic of DsbG may allow the enzyme to bind its substrates more efficiently than DsbC, since the preferred substrates of DsbG are folded proteins with an oxidized single cysteine (DEPUYDT *et al.* 2009).

1.2.1.5. DsbD: a thiol-disulfide transporter maintains both DsbC and DsbG in the reduced active state

Both periplasmic DsbC and DsbG must be maintained in the reduced state to be active. This is achieved by the inner membrane protein DsbD (BESSETTE *et al.* 1999; MISSIAKAS *et al.* 1995; RIETSCH *et al.* 1997). In *dsbD* mutants, both DsbC and DsbG remain oxidized and therefore can no longer act as isomerases or reductases of misoxidized proteins. Upon reduction of DsbC and DsbG, DsbD becomes oxidized. The oxidized DsbD is then recycled to its reduced form by accepting electrons from the thioredoxin/thioredoxin reductase system in the cytoplasm. Reducing power to maintain DsbC and DsbG in their reduced state originates from NADPH by transferring electrons to thioredoxin, via thioredoxin reductase, which in turn will reduce DsbD. DsbC and DsbG are kept reduced by DsbD at the expense of NADPH oxidation in the cytoplasm (RIETSCH *et al.* 1996; RIETSCH *et al.* 1997).

DsbD is 59 kDa protein containing three domains: an N-terminal periplasmic domain (DsbDα) with an immunoglobulin-like fold (HAEBEL *et al.* 2002), a hydrophobic core

domain (DsbDβ) with eight transmembrane segments (CHO *et al.* 2007) and a C-terminal periplasmic domain (DsbDγ) with a thioredoxin-like fold (ROZHKOVA *et al.* 2004). Each domain has a pair of cysteines that is essential for the protein's activity (KATZEN and BECKWITH 2000). A set of *in vitro* (COLLET *et al.* 2002; HAEBEL *et al.* 2002; ROZHKOVA *et al.* 2004) and *in vivo* (KATZEN and BECKWITH 2000) experiments demonstrated that DsbD transfers electrons across the membrane by a cascade of thiol-disulfide exchange reactions. Electrons are transferred from the cytoplasmic Trx to the cysteine residues of DsbDβ, then successively to the cysteines of DsbDγ and DsbDα and finally to the catalytic site of DsbC and/or DsbG.

The standard redox potentials of their active-site cysteine pairs are -270 mV (Trx), -246 mV (DsbDβ), -241 mV (DsbDγ), -229 mV (DsbDα), -130 mV (DsbC) and/or -126 mV (DsbG) (COLLET *et al.* 2002; ROZHKOVA and GLOCKSHUBER 2008). Thus, electron-transfer reactions between every two active-site cysteine pairs along the DsbD-mediated electron flow are thermodynamically favorable (COLLET *et al.* 2002; ROZHKOVA and GLOCKSHUBER 2008).

The mechanism by which the two cysteines of DsbDβ transport electrons across the membrane is far from being understood but recent studies suggest that DsbD adopts an hourglass-like structure. DsbDβ forms two cavities accessible to one or the other side of the membrane and the two active cysteines are localized at the constriction site (CHO and BECKWITH 2006; CHO and BECKWITH 2009; CHO *et al.* 2007). This hourglass-like arrangement appears to allow two cysteines of DsbDβ to interact with the cysteines of thioredoxin on the cytoplasmic side and with the cysteines of DsbDγ on the periplasmic

side of the membrane and facilitates the electron transfer reaction across the membrane. DsbD is a unique "membrane transporter of reducing power" by carrying two electrons across the inner membrane from one thioredoxin on one side of the membrane to another thioredoxin on the opposite side. The physical arrangement of DsbD raises one question, how the transfer of electrons across the membrane can occur without perturbing the function of the membrane as a permeation barrier? The determination of the three-dimensional structure of DsbDβ may be necessary to answer this question. In summary, the oxidative folding of substrate proteins is achieved by a set of thioredoxin-like proteins with different redox properties. The cooperation of oxidizing and isomerizing activities is fundamental for the functionality of many proteins of the periplasm.

1.2.2. Disulfide bond formation in Gram-positive bacteria

While disulfide bond formation has been intensively studied in Gram-negative bacteria, a similar pathway has emerged in Gram-positive bacteria. *Bacillus subtilis* has been mostly used to study disulfide bond formation in Gram-positive bacteria. *B. subtilis* has a set of proteins called Bacillus disulfide bond (Bdb), reminiscent of the *E.coli* Dsb proteins.

BdbD is the functional equivalent of DsbA and introduces disulfide bonds. BdbD is anchored to the cytoplasmic membrane and adopts a Trx-fold containing a CPSC catalytic motif, with a redox potential of -75 mV. This redox potential is remarkably oxidizing (CROW *et al.* 2009). The structure of BdbD revealed the unexpected presence of a calcium-binding site influencing the redox properties of the protein. In absence of calcium, the redox potential of BdbD decreases by 20 mV (MEIMA *et al.* 2002). BdbD is

reoxidized by BdbC, which is encoded by the same bicistronic operon (BOLHUIS *et al.* 1999; MEIMA *et al.* 2002). BdbC displays 40% identity with DsbB and is predicted to have the same topology. BdbC, like DsbB, also has two extracytoplasmic loops, each containing a pair of catalytic cysteine residues, found in a C*XX*C motif.

In contrast to *E.coli*, disulfide isomerases have so far remained undetected in *B. subtilis* (KOUWEN and VAN DIJL 2009). Indeed, *B. subtilis* lacks obvious orthologs of *E. coli* isomerases such as DsbC and DsbG. The *B. subtilis* system for protein disulfide isomerization has not been identified yet.

A reduction pathway also has been identified in *B. subtilis* and is composed of CcdA and ResA. CcdA is a membrane protein with six transmembrane domains that transports reducing equivalents across the inner membrane from the cytoplasm to the extracytoplasmic compartment. CcdA corresponds to the DsbDβ and lacks the periplasmic DsbDα and DsbDγ domains (KATZEN *et al.* 2002; SCHIÖTT *et al.* 1997b). *B. subtilis* CcdA most likely accepts electrons from a thioredoxin in the cytoplasm and transfers them to a thiol-disulfide oxidoreductase on the outer side of the membrane to reduce disulfide bonds of target proteins. ResA, a thiol-disulfide oxidoreductase, is thought to be reduced by CcdA (ERLENDSSON *et al.* 2003) and can be seen as corresponding to the γ domain of DsbD. *B. subtilis* CcdA (cytochrome *c* deficiency protein A) was first identified in the context of cytochrome *c* maturation. Indeed, *B. subtilis* CcdA-defective mutants are impaired for cytochrome *c* production (SCHIÖTT *et al.* 1997a; SCHIÖTT *et al.* 1997b). In addition, *B. subtilis* ResA, a thiol-disulfide oxidoreductase, believed to be reduced by CcdA is also involved in cytochrome *c*

maturation (COLBERT *et al.* 2006; ERLENDSSON *et al.* 2003). Similary to *E. coli* reduction pathway composed of DsbD and CcmG, the reduction pathway, in *B. subtilis*, is composed of CcdA and ResA, their functional counterparts. DsbD reduces CcmG (CHUNG *et al.* 2000; KADOKURA *et al.* 2003; KATZEN and BECKWITH 2000; KRUPP *et al.* 2001), a membrane-tethered thioredoxin-like protein involved in the reduction of apocytochromes *c*. Indeed, both proteins are essential for cytochrome *c* maturation, as their respective *E. coli* mutant strains are deficient for cytochrome *c* assembly (FABIANEK *et al.* 1998; KATZEN and BECKWITH 2000; KATZEN *et al.* 2002; REID *et al.* 2001; STEWART *et al.* 1999; STIRNIMANN *et al.* 2006).

The involvement of DsbD/CcdA, CcmG and ResA in the reduction of apocytochromes *c*, a chemical requirement in the assembly process will be discussed in detail in section 1.3.

1.2.3. Vitamin K epoxide reductase as a substitute for DsbB

Recent bioinformatic studies revealed that some bacteria possess DsbA-like proteins but lack DsbB orthologs (DUTTON *et al.* 2008). This finding led to the identification of a new protein with a DsbB-like activity in the predicted proteomes of several bacteria such as actinobacteria (*e.g. Myboacterium tuberculosis*), and aerobic δ-proteobacter (*e.g. Desulfibrio*) (DUTTON *et al.* 2008). This protein is the bacterial ortholog of the vitamin K epoxide reductase (VKOR), an integral membrane protein at the endoplasmic reticulum (ER) (DUTTON *et al.* 2008). VKOR catalyzes the reduction of vitamin K epoxide into reduced vitamin K (GOODSTADT and PONTING 2004; LI *et al.* 2004). Reduced vitamin K is required as a cofactor for the γ-carboxylation of clotting factors in blood (TIE and

STAFFORD 2008). The reduction of vitamin K is one of the enzymatic reactions leading to blood coagulation in humans. Human VKOR reduces vitamin K epoxide via four conserved catalytic cysteine residues, two of them found in a C*XX*C motif (CROW *et al.* 2009; ROST *et al.* 2005). Human VKOR has been found to interact with a membrane anchored thioredoxin-like (TMX) that operates in sulfhydryl oxidation in the ER lumen. Based on this interaction, VKOR and TMX were proposed to define a disulfide bond forming pathway where TMX catalyzes disulfide bond formation in the ER lumen and is recycled back to its oxidized form by VKOR with vitamin K epoxide as a final electron acceptor (SCHULMAN *et al.* 2011). Because vitamin K is a quinone, TMX/VKOR can be viewed as equivalent to the DsbA/DsbB system.

A similar pathway seems to operate in some bacteria lacking DsbB orthologs. Disulfide bond formation can be restored by expressing *Mycobacterium tuberculosis* VKOR-like protein in an *E.coli dsbB*-null (WANG *et al.* 2011). Although bacterial VKOR-like proteins do not show sequence similarity to DsbB, these results suggest that they may be carrying out similar reactions to those catalyzed by DsbB, *i.e.* the oxidation of DsbA-like proteins followed by the reduction of quinones. Indeed, the heterologous functional complementation of *ΔdsbB* by bacterial VKOR-like is dependent upon DsbA, suggesting that VKOR is functionally equivalent to DsbB (DUTTON *et al.* 2008; DUTTON *et al.* 2010). In some bacteria, such as cyanobacteria, lacking both DsbA and DsbB, the C-terminal domain of VKOR-like is fused to a thioredoxin-like domain that is functionally equivalent to DsbA (SINGH *et al.* 2008). The structure of cyanobacterial VKOR-like has been solved and the protein core appears as a four transmembrane domain surrounding a

quinone that acts as an acceptor in a disulfide bond forming cascade (LI *et al.* 2010b). Based on these results, the mode of action of VKOR was proposed to be very similar to that of DsbB. Both proteins catalyze disulfide bond formation followed by the reduction of a quinone/menaquinone in the case of DsbB or vitamin K in the case of VKOR (BADER *et al.* 1999; DUTTON *et al.* 2008).

1.2.4. Disulfide bond formation in mitochondria

While the presence of a thiol-metabolizing pathway is well established in bacteria, there was little support for the operation of thiol-based chemistry in the mitochondrial intermembrane space (IMS), which is topologically equivalent to the bacterial periplasm. The IMS is one aqueous subcompartment delimited by two membranes, the outer and the inner mitochondrial membranes. A second aqueous subcompartment is the matrix surrounded by the inner membrane (see Figure 1.4). The IMS of mitochondria harbors proteins crucial for a variety of fundamental processes of the cell. These proteins are involved in the production of energy by oxidative phosphorylation, in the transport of proteins, metabolites, metal ions, in apoptotic processes and in the detoxification of harmful reaction products (HELL 2007). Many of these IMS resident proteins contain disulfide bonds. The disulfide bond in the Rieske iron-sulfur protein, a component of the respiratory electron chain, was identified more than a decade ago (FIELD *et al.* 2003; IWATA *et al.* 1996). Other disulfide bonded proteins in the IMS include Cox 11, a copper-binding protein required for Cu incorporation into the Cu(B) site of cytochrome *c* oxidase (BANCI *et al.* 2004), Cox 12, a structural subunit of cytochrome *c* oxidase

(TSUKIHARA *et al.* 1996), Cox 17 (ARNESANO *et al.* 2005), Qrc8, a structural subunit of the bc_1 complex (IWATA *et al.* 1998), Sco1, a thioredoxin-like protein involved in cytochrome *c* oxidase assembly (BALATRI *et al.* 2003) and the small Tim involved in protein import. The occurence of disulfide bonded proteins in the IMS suggests that a disulfide bond forming system must also operate in this compartment. The system must be different from the bacterial DsbAB since no orthologs are found in this compartment. Indeed, Mesecke *et al.* discovered a new machinery catalyzing the oxidation of proteins in the IMS (MESECKE *et al.* 2005). This machinery is composed of two essential components Mia40 and Erv1, and constitutes a disulfide relay system because oxidation of substrate proteins is functionally and temporally coupled to their import into the IMS (see Figure 1.4). Only reduced and unfolded proteins can pass through the import pore of the mitochondrial outer membrane also called translocon of the outer membrane or TOM complex. Interestingly, the cysteine residues of the substrate proteins of the Mia40/Erv1 system form characteristic patterns, called twin CX_3C and twin CX_9C motifs. These motifs form helix-loop-helix structures. Each helix contains a pair of cysteine residues spaced by either three or nine amino acids. The two central and the two distal cysteine residues are connected by disulfide bonds, locking the helices in an antiparallel orientation.

1.2.4.1. Mia40: the redox-regulated import receptor

The import receptor Mia40 (mitochondrial import and assembly) is a unique oxidase ubiquitously present in eukaryotes. Mia40 is the central player of the mitochondrial

disulfide formation machinery and characterized by a highly conserved cysteine-containing domain (CHACINSKA *et al.* 2004; NAOE *et al.* 2004; TERZIYSKA *et al.* 2005). In fungi, this domain is tethered to the inner membrane *via* a N-terminal transmembrane anchor (NAOE *et al.* 2004; TERZIYSKA *et al.* 2005) whereas Mia40 orthologs in animals and plants are soluble in the IMS (HOFMANN *et al.* 2005). The conserved Mia40 domain consists of about 60 amino acid residues with six essential cysteine residues. The cysteine residues form an invariant CPC signature followed by a twin CX_9C motif similar to the one found in many Mia40 substrates. Based on *in vitro* experiments with the purified conserved domain of Mia40, it is proposed that the cysteine residues of the CPC motif represent the redox-active thiol group. There is no evidence whether the two CX_9C motifs are involved in protein structure or part of the active site for the oxidation of substrate proteins (GRUMBT *et al.* 2007). Mia40 serves as a receptor by directly interacting with newly synthesized CX_3C and CX_9C proteins and facilitates their uptake into the mitochondrial IMS. Mia40 forms a stable but transient mixed disulfide with the imported protein (CHACINSKA *et al.* 2004; MESECKE *et al.* 2005; MULLER *et al.* 2008).

1.2.4.2. Erv1: a sulfhydryl oxidase in the IMS

After the transfer of its disulfide to substrate proteins, Mia40 is reoxidized by Erv1 (Essential for Respiration and Vegetative growth), a soluble homodimeric flavoprotein in the IMS (BIEN *et al.*; LEE *et al.* 2000; LISOWSKY 1992; MESECKE *et al.* 2005). Erv1-like proteins are ubiquitously present in mitochondria and mitosomes of eukaryotes (BURRI *et al.* 2006).

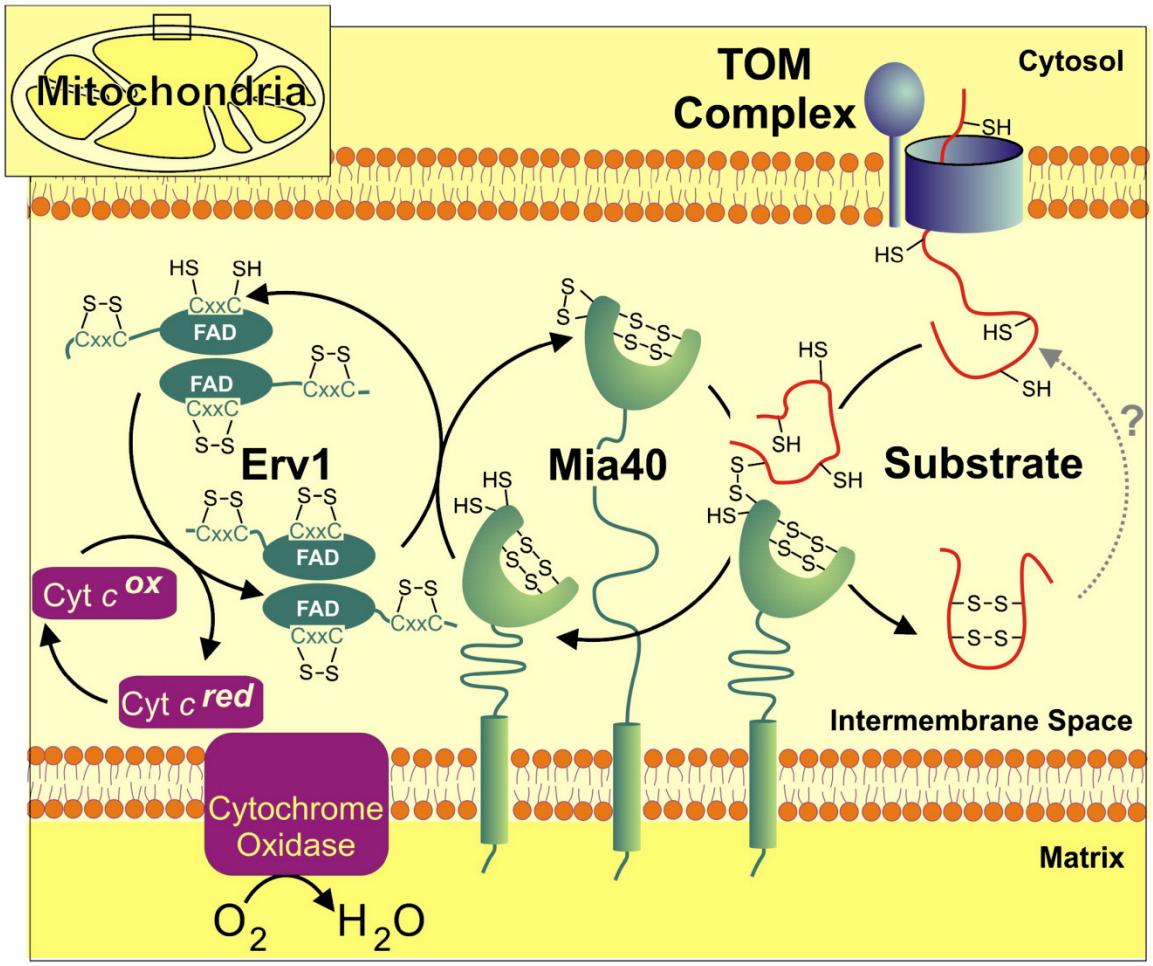


Figure 1.4. Protein oxidation in mitochondria.

Newly synthesized proteins enter the IMS through the protein-conducting channel of the TOM complex. In the IMS they transiently bind to Mia40 before they are released in an oxidized, stably folded form. During the import reaction, Mia40 is reduced. Subsequently, Mia40 is reoxidized by Erv1. *In vitro*, Erv1 can receive electrons from Mia40 and passes them via FAD onto molecular oxygen giving rise to the production of hydrogen peroxide. *In vivo*, however, electrons are delivered from the flavin cofactor of Erv1 to cytochrome *c* and further into the respiratory chain which produces water. This connection to the respiratory chain increases the efficiency of the re-oxidation of Mia40 and prevents the formation of potentially harmful hydrogen peroxide. Source: (HERRMANN *et al.* 2009).

Erv1 is composed of a catalytic domain containing an essential redox-active C*XX*C motif adjacent to the isoalloxazine ring of FAD that allows the efficient electron transfer from

the pair of cysteines to the FAD cofactor (GROSS *et al.* 2002; STEIN and LISOWSKY 1998; VITU *et al.* 2006). The FAD domain can catalyze the transfer of electrons from thiol groups to oxygen and produce hydrogen peroxide, a reactive oxygen species (ROS). The FAD domain can also reduce another electron acceptor such as cytochrome *c* (BIHLMAIER *et al.* 2007; DABIR *et al.* 2007). Erv1 delivers electrons to the respiratory chain via cytochrome *c* and the cytochrome oxidase produces water from oxygen (see Figure 1.4). This interaction with the respiratory chain not only makes the re-oxidation of Mia40 much more efficient, even at low oxygen concentrations, but also prevents the formation of hydrogen peroxide, potentially harmful for living cells (ALLEN *et al.* 2005; BIHLMAIER *et al.* 2007; DABIR *et al.* 2007; FARRELL and THORPE 2005).

1.3. *c*-type cytochrome biogenesis

1.3.1. Cytochrome *c* definition

The *c*-type cytochromes, also generically referred to as cytochromes *c* are heme containing proteins commonly involved in electron transfer reactions. Their redox activity is determined by the change of valence of the iron atom coordinated to the four nitrogen atoms of the heme co-factor. The reduction of ferric ion (Fe^{3+}) to the ferrous form (Fe^{2+}) allows the transfer of a single electron at a time (BONNARD *et al.* 2010; FERGUSON *et al.* 2008; THÖNY-MEYER 2002). Cytochromes *c* occur as soluble or membrane-bound molecules that function as electron carriers between enzymatic complexes involved in energy transduction processes such as respiration and photosynthesis. The typical purpose of the electron transport is then to generate a proton

gradient across the inner membrane. To finalize the energy conversion, this gradient is in turn used to produce ATP. Hence, cytochromes *c* occur in all energy-transducing membrane systems such as the ones found in bacteria, mitochondria and chloroplasts. In some eukaryotes, cytochromes *c* contribute to signaling events that lead to apoptosis or programmed cell death (JIANG and WANG 2004).

The *c*-type cytochromes are characterized by the covalent attachment of heme (Fe-protoporphyrin IX) to the apoprotein via two thioether linkages between the vinyl side chains of the prosthetic group and cysteine thiols of the polypeptide (carbon-sulfur bonds). While bacterial cytochromes *c* can contain from one to up 27 hemes, mitochondrial and chloroplast cytochromes *c* are monoheme molecules (BONNARD *et al.* 2010; SHI *et al.* 2007). The two cysteines almost always occur in a conserved C*XX*CH, where the histidine is an axial ligand to the heme iron and *X* can be any residue (see Figure 1.5). C*XXX*CH, C*XXXX*CH, $C(X)_{15}CH$ and C*XX*CK motifs are variations to the canonical heme binding site that are found in some bacterial cytochromes *c* (HARTSHORNE *et al.* 2007; HARTSHORNE *et al.* 2006; JUNGST *et al.* 1991; RIOS-VELAZQUEZ *et al.* 2001). Another heme binding site variation occurs in the phylum Euglenozoa, including *Trypanosoma*, *Leishmania* and *Euglena* species, where heme is attached via a single thioether bond at a F/A*XX*CH motif in mitochondrial *c*-type cytochromes (ALLEN *et al.* 2004).

At the C*XX*CH heme binding site, the first cysteine of the motif always forms a stereospecific link with the side chain at the C3 position on the porphyrin ring while the second cysteine is invariably bonded to the side chain at the C8 position (see Figure 1.5).

By analogy to the C*XX*CH motif, the cysteine in the F/A*XX*CH heme binding motif appears to be bonded to the vinyl at the C8 position. Despite the difference in heme attachment in Euglenozoa (*i.e.* one thioether bond instead of two in the typical C*XX*CH motif), the structure of cytochromes *c* and the stereochemistry of the covalent heme attachment to the protein are remarkably very similar (FULOP *et al.* 2009). Interestingly, *in vitro* reconstitution of the heme ligation reaction shows that the vinyl in C3 is attached to a C*XX*AH apocytochrome *c* variant while the vinyl in C8 is linked to a A*XX*CH variant, an indication that the chemistry of heme attachment dictates the stereospecificity (DALTROP *et al.* 2003).

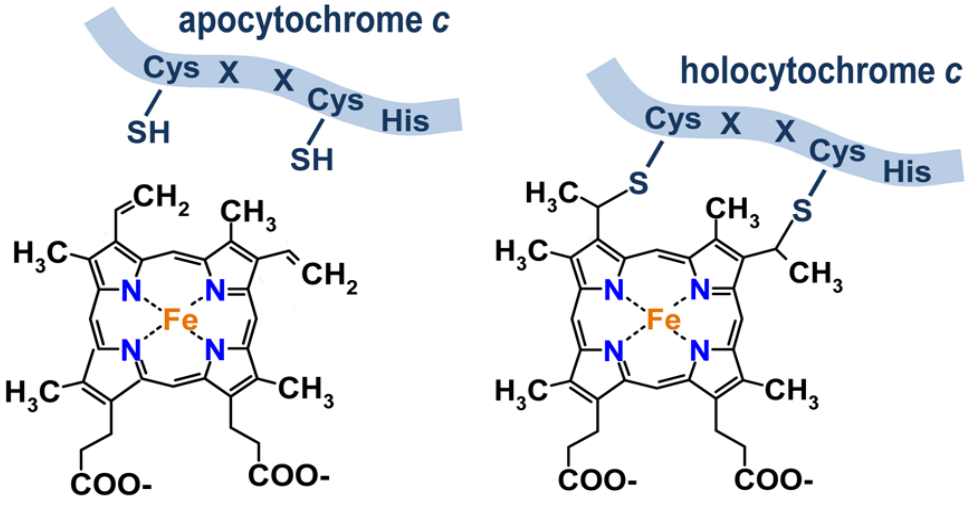


Figure 1.5. Stereospecific heme attachment reaction.

The lyase is the enzyme responsible for the stereospecific addition of the cysteinyl thiols from the apocytochrome C*XX*CH motif (shown in blue) to the α carbons at the vinyl group in positions C3 and C8 of Fe-protoporphyrin IX (heme, shown in black with iron in orange and nitrogen in blue). This reaction occurs on the *p*-side of the energy-transducing membranes. The numbering of the carbons atoms in the porphyrin rings follows the rules

continued

Figure 1.5. continued.
of the International Union of Pure and Applied Chemistry (IUPAC). Source: (HAMEL *et al.* 2009).

1.3.2. *c*-type cytochromes biogenesis is a multiple step process

The biogenesis of *c*-type cytochromes occurs in evolutionary related compartments, the bacterial periplasm, the mitochondrial IMS and the thylakoid lumen (in chloroplasts or cyanobacteria), which are referred to as the positive side (*p*-side) of the energy transducing membrane. Because the site of assembly of cytochromes *c* is distinct from the sites of heme cofactor and apoprotein synthesis, their biosynthesis follow a complex pathway with multiple steps: 1) apocytochromes *c* synthesis and translocation across a lipid bilayer to the *p*-side where cytochromes *c* are assembled and function, 2) synthesis and transport of heme, (translocation of apocytochromes *c* and transport of heme take place via distinct and independent mechanisms), 3) transfer of reducing equivalents across the membrane to maintain heme and the sulfhydryl groups of the C*XX*CH motif under a reduced state and 4) stereospecific attachment of the heme cofactor to the apocytochromes (BONNARD *et al.* 2010; CRAMER *et al.* 1994; HAMEL *et al.* 2009; KRANZ *et al.* 1998; KRANZ *et al.* 2009; NAKAMOTO *et al.* 2000; PAGE *et al.* 1998; SANDERS *et al.* 2010; THÖNY-MEYER 1997; THÖNY-MEYER 2002; XIE and MERCHANT 1998).

1.3.3. Redox chemistry in the context of cytochrome *c* assembly

Synthesis of holocytochrome *c* requires reducing conditions. The implication of redox chemistry in the assembly process was first established through pioneer work in fungal

mitochondria. *In organello* reconstitution of holocytochrome *c* and c_1 assembly indicates that both heme and apocytochrome *c* need to be reduced prior to the heme ligation reaction catalyzed via their respective heme lyases (BASILE *et al.* 1980; NICHOLSON and NEUPERT 1989; NICHOLSON *et al.* 1989; TONG and MARGOLIASH 1998).

In vitro reconstitution of *c*-type cytochrome with reduced apocytochrome and ferrous heme (Fe^{2+}) but not ferric (Fe^{3+}) as substrates, confirms the chemical need of a reductant for both the co-factor and the apoprotein. The reason for this requirement in the case of heme is not entirely clear but it appears that the vinyl groups can only be chemically reactive for thioether bond formation when heme is provided under the reduced form and not under the oxidized form (DALTROP *et al.* 2002; DALTROP and FERGUSON 2003). Reducing conditions are also needed to maintain the cysteines of the C*XX*CH heme binding site under a reduced form (*e.g.* sulfhydryl) (DALTROP *et al.* 2002; DALTROP and FERGUSON 2003).

In vivo, the involvement of redox chemistry for cytochrome *c* maturation was first established in bacteria. It is now obvious that independent pathways controlling the redox state of heme and C*XX*CH sulfhydryls are required for cytochrome c assembly (see section 1.3.4). While the operation of a disulfide-reducing pathway for the maintenance of the C*XX*CH is now well documented, the pathways controlling the redox state of heme are only beginning to emerge (see section 1.3.4).

The bacterial periplasm is the compartment where *c*-type cytochromes are assembled but it also houses the Dsb/Bdb system controlling the oxidative folding of cysteine-containing proteins (see sections 1.2.1 and 1.2.2). The current model is that

apocytochrome *c* is first a substrate of the Dsb/Bdb machinery, which introduces intramolecular disulfides between the two cysteines of the C*XX*CH motif and needs to be consequently reduced by a dedicated disulfide-reducing pathway to provide free sulfhydryls for the heme ligation reaction. It was initially postulated that disulfide bond formation at the C*XX*CH was a required step to ensure stereospecific attachment of heme to apocytochromes *c* (SAMBONGI and FERGUSON 1996). This statement was based on the observation that loss of DsbA/DsbB results in cytochrome *c* deficiency and that the provision of oxidized thiols to *dsbA*/*dsbB* mutants rescues the cytochrome *c* maturation defect (METHERINGHAM *et al.* 1995; METHERINGHAM *et al.* 1996; SAMBONGI and FERGUSON 1996). This postulate has since been challenged as multiple experimental evidences indicate that the disulfide bond forming catalysts are not essential for cytochrome *c* maturation (ALLEN *et al.* 2003b; DESHMUKH *et al.* 2000; ERLENDSSON *et al.* 2003; FEISSNER *et al.* 2005; KRANZ *et al.* 2002). However, the fact that *dsbAB* mutants accumulate reduced levels of holocytochrome *c* suggests that the disulfide bond forming machinery might be necessary for optimal production of *c*-type cytochromes, at least in *Rhodobacter* (TURKARSLAN *et al.* 2008). The operation of a disulfide-reducing pathway for cytochrome *c* maturation will be discussed in section 1.3.4.

1.3.4. Three different systems for cytochrome *c* assembly

The cytochrome *c* maturation is a multiple step process by which cells converts a linear apocytochrome into a three dimensionally structured polypeptide that contains one or more covalently bound heme cofactors. Surprisingly, three different systems (system I,

system II, system III) exist to achieve this post-translational modification. This is a rather surprising finding as the attachment of heme to a C*XX*CH motif appears to be, on the surface, a simple chemical reaction. These systems operate in prokaryotes (bacterial cytoplasmic membrane and cyanobacterial thylakoid membrane) and eukaryotic organelles (mitochondrial inner membrane and chloroplast thylakoid membrane). Their distribution does not appear to follow the evolutionary tree of life and lateral transfer of biogenesis pathways has been proposed (ALLEN *et al.* 2008; BERTINI *et al.* 2007; GOLDMAN and KRANZ 1998).

System I is present in α- and γ-proteobacteria, deinoccoci, archaea, plant and some protozoal mitochondria. System II is found in Gram-positive bacteria, cyanobacteria, plastids and in most of the β-, δ-, and ε-proteobacteria, while system III is restricted to the mitochondria of fungal, animal and some protozoa such as green algae and paramecia.

A newly identified system IV also called CCB pathway, C for Cofactor binding, C for cytochrome b_6f complex and B for PetB subunit which is cytochrome b_6, is required for the covalent attachment of heme c_i to cytochrome b_6 of the cytochrome b_6f complex present in photosynthetic eukaryotes and cyanobacteria (DE VITRY 2011; KURAS *et al.* 1997; KURAS *et al.* 2007; LYSKA *et al.* 2007). Cytochrome b_6 is a tri-heme cytochrome with two *b*-type hemes and one heme c_i. Heme c_i is attached to cytochrome b_6 via a single thioether bond. The cysteine involved in the thioether bond is not found in a *XXX*CH heme binding motif and no amino acid acts as axial ligand of the iron atom. In addition, the heme c_i is located on the *n*-side of the thylakoid membrane (DE VITRY 2011; KURISU

et al. 2003; STROEBEL *et al.* 2003). Therefore, the CCB pathway is very distinct from system I, II and III.

Because the prototypical components of systems I, II or III are not present in the predicted proteomes of euglenozoa that possess mitochondrial cytochromes *c* with a A/FXXCH motif, the operation of a novel assembly pathway (system V) has been postulated (ALLEN 2011).

1.3.4.1. System I/CCM

System I, also referred to as the CCM pathway (for Cytochrome *C* Maturation), has been initially discovered in α- and γ-proteobacteria microbial models such as *Rhodobacter capsulatus* (see Figure 1.6) and *Escherichia coli* (see Figure 1.7), respectively. The typical cytochrome *c* assembly machinery of system I is composed of 9 membrane and periplasmic assembly factors (CcmA to CcmH and DsbD/CcdA) (see Table 1.1). In some bacteria, like *Rhodobacter capsulatus*, the C-terminal of CcmH occurs as a separate protein (CcmI-2) (see Figure 1.6). A detailed view of the bacterial system I apparatus, for cytochrome *c* maturation, has now emerged and the assembly factors can be functionally divided in three categories: 1) a heme delivery system, 2) a transmembrane thio-reduction pathway and 3) an apocytochrome *c* heme lyase.

1) A heme delivery system (CcmABCDE)

Heme is synthesized in the bacterial cytoplasm and must be delivered to the other side of the membrane. CcmABCDE proteins define a heme delivery pathway to the heme lyase

unit composed of CcmFH. The current working model for CcmABCDE is as follows: CcmC loads heme onto CcmE and forms a complex that is stabilized by CcmD. CcmAB, an ATP binding cassette (ABC)-type transporter releases the heme containing form of CcmE (holoCcmE) from CcmC. CcmE is a periplasmic protein that binds heme via a covalent adduct between one vinyl of heme and a conserved histidine residue. Alteration of the conserved histidine in CcmE not only prevents the covalent attachment of heme, but also blocks *c*-type cytochrome synthesis (SCHULZ *et al.* 1998). CcmE first interacts with CcmC to capture heme and with CcmF for covalent heme attachment to apocytochrome *c* (REN *et al.* 2002; REN and THONY-MEYER 2001). CcmE is viewed as a heme relay molecule because heme is first bound to CcmE and then transferred for attachment to the apocytochrome *c* substrate (SCHULZ *et al.* 1998). The mechanism by which the covalent bond between heme and CcmE is resolved remains unknown (MAVRIDOU *et al.* 2012). CcmC is a polytopic membrane protein with a hydrophobic WWD domain in a periplasmic loop. The protein binds heme via the WWD motif and flanking histidine ligands on adjacent periplasmic loops. The WWD motif and the histidine ligands are proposed to define the external heme binding domain of CcmC. CcmC is necessary and sufficient for heme attachement onto CcmE. When holoCcmE is formed, heme is still coordinated by CcmC histidines that provide the fifth and sixth axial ligands to the heme iron. CcmD, a small integral membrane protein mediates and stabilizes the complex formation between CcmC and CcmE (AHUJA and THÖNY-MEYER 2005; SCHULZ *et al.* 1999).

CcmA, a soluble ATPase with the characteristic ATP binding domain (Walker A and B motifs) interacts with CcmB, an integral membrane protein with six transmembrane helices to form the core of an ABC transporter. CcmA and CcmB were initially postulated to act as a heme transporter but this role now appears unlikely because heme remains attached to CcmE when the ATPase activity of CcmAB is lost or in a *ccmAB* mutant (CHRISTENSEN *et al.* 2007; FEISSNER *et al.* 2006a). The current view is that CcmAB acts by releasing holoCcmE from CcmC by an ATP dependent process (CHRISTENSEN *et al.* 2007; FEISSNER *et al.* 2006a). The mechanism by which heme is transported across the membrane remains unknown and CcmC has been proposed to be involved in this function (KRANZ *et al.* 2009; REN and THONY-MEYER 2001; RICHARD-FOGAL and KRANZ 2010).

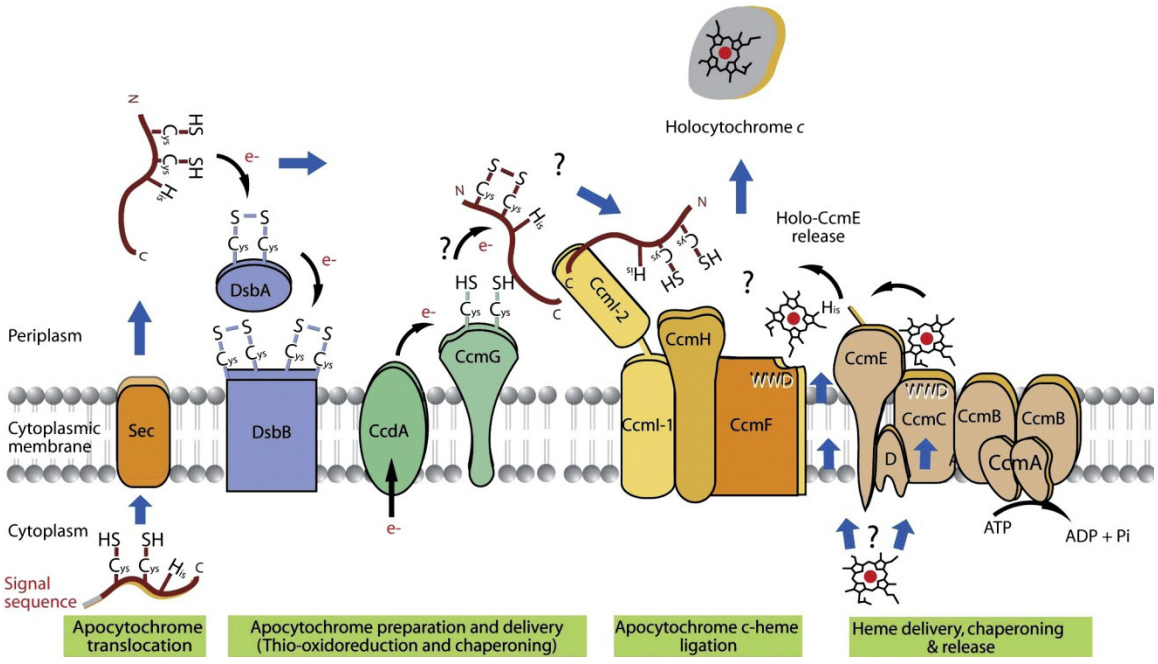


Figure 1.6. Cytochrome *c* maturation (Ccm) system in *R. capsulatus.*

continued

Figure 1.6. continued.
The cytochrome *c* maturation is a multiple steps process. The first step is the transport of heme and its preparation for ligation to apocytochrome and involves CcmABCD proteins which form an ABC-type transporter that has a role in the delivery of heme to the heme chaperone CcmE before the ligation. It is unknown whether heme is transported via CcmABCD complex or by another unknown protein. CcmA and CcmB are required for the release of heme-bound CcmE from CcmC and CcmD. CcmC and CcmD are involved in the heme attachment to CcmE. The second step involves the apocytochrome thioredox and chaperoning processes. After the translocation of apocytochrome into the periplasm, the cysteine thiols are first oxidized by DsbA-DsbB, then reduced by CcdA, CcmG and/or CcmH. The third step is the heme ligation by the core complex CcmHIF. CcmI binds to C-terminal part of apocytochrome to deliver it to the core complex for the catalysis of the thioether bond formation between reduced apocytochrome and heme vinyl groups forming the mature cytochrome *c*. Source: (EKICI *et al.* 2011).

2) A transmembrane thio-reduction pathway

Apocytochromes *c* are translocated to the periplasm via the SecYEG secretory pathway (see Figure 1.6). The bacterial Sec system is composed of SecY, SecE and SecG proteins that form a channel in the inner membrane. Through the channel, SecA, a cytoplasmic motor protein drives the protein precursors to the periplasm. This active transport is ATP dependent. The narrow diameter of the channel only allows the transport of unfolded or very loosely folded proteins (WICKNER and SCHEKMAN 2005). Upon translocation across the cytoplasmic membrane, the DsbA-DsbB dependent oxidative protein folding pathway is believed to rapidly oxidize the thiol groups of apocytochromes *c* (KADOKURA *et al.* 2003; NAKAMOTO and BARDWELL 2004). The disulfide bond formed between the cysteines at the C*XX*CH heme binding motif needs to be consequently reduced to provide free sulfhydryls for the heme attachment. A trans-membrane thioreduction pathway involving DsbD (or CcdA), CcmG and CcmH has been proposed to operate in the delivery of reductants for the maintenance of the C*XX*CH sulfhydryls. Bacterial

dsbD/*ccdA*, *ccmG* and *ccmH* mutants are defective for cytochrome *c* assembly showing that each component is essential for this process (ALLEN *et al.* 2003b; FABIANEK *et al.* 2000; KADOKURA *et al.* 2003; MÖLLER and HEDERSTEDT 2006; RITZ and BECKWITH 2001).

DsbD/CcdA is a thiol disulfide transporter with a central role in redox relay (KOUWEN and VAN DIJL 2009; MÖLLER and HEDERSTEDT 2006; STIRNIMANN *et al.* 2006). DsbD and CcdA are polytopic membrane proteins with conserved within-membrane cysteines involved in transducing the reducing power across the membrane (KOUWEN and VAN DIJL 2009; MÖLLER and HEDERSTEDT 2006; STIRNIMANN *et al.* 2006). While DsbD and CcdA differ in their N and C-terminal extensions in the periplasm, they display a common central membrane domain with the redox active cysteines (see sections 1.2.1.5 and 1.2.2). CcdA or DsbD can occur in system I or system II bacteria and the proteins are functionally interchangeable (KATZEN *et al.* 2002). CcmG and CcmH are membrane-anchored oxidoreductases with a single C*XX*C domain facing the periplasm, which are believed to participate along with CcdA/DsbD in a cascade for the reduction of a disulfide bond at the C*XX*CH heme binding site. Two models for the transfer of reducing equivalents to apocytochromes *c* have been proposed. Both models postulate the operation of sequential thiol-disulfide exchange reactions involving Ccda/DsbD, CcmG and CcmH. In the first one, the electrons conveyed by CcdA/DsbD are sequentially transferred to CcmG, CcmH to reduce a disulfide in the apocytochrome *c* C*XX*CH motif (FABIANEK *et al.* 1999). In the second model, CcmH and the apocytochrome *c* form a mixed disulfide that is resolved by CcmG, resulting in a reduced apocytochrome and a

mixed disulfide between CcmG and CcmH. The mixed disulfide was proposed to be reduced by CcdA/DsbD (REID *et al.* 2001). The first model is supported by midpoint potential determination and redox assays using recombinant forms of CcmG and CcmH (MONIKA *et al.* 1997; SETTERDAHL *et al.* 2000). *In vitro* thiol-disulfide exchange reactions demonstrate that CcmG can reduce oxidized CcmH while CcmH is able to reduce a disulfide in an apocytochrome *c* peptide (MONIKA *et al.* 1997). *In vivo*, while a mixed disulfide between the thiol-disulfide transporter and CcmG has been detected, there is still no evidence that CcmG is the reductant of CcmH and that apocytochrome *c* is reduced by CcmH (KATZEN *et al.* 2002; STIRNIMANN *et al.* 2005).

Recent biochemical analyses of CcmG and CcmH soluble forms suggest that CcmG acts as a reductant to resolve a mixed disulfide betwee apocytochrome *c* and CcmH (DI MATTEO *et al.* 2010; DI MATTEO *et al.* 2007). While it seems that CcmH directly reduces a disulfide in apocytochrome c, experimental validation *in vivo* is required to establish if CcmH is reduced by CcmG when forming an intramolecular disulfide or an intermolecular disulfide with apocytochrome *c*. Interestingly, the tertiary structure of CcmG shows a canonical thioredoxin fold and a cavity that could act as a binding site for apocytochrome *c* (DI MATTEO *et al.* 2010; OUYANG *et al.* 2006). This is consistent with the putative apocytochrome *c* chaperoning function that was evidenced for *R. capsulatus* CcmG (TURKARSLAN *et al.* 2008). The structure of CcmH positions the RCXXC motif in a three-helix bundle that is not typical of a thioredoxin-like fold, further underscoring the uniqueness of this redox molecule (DI MATTEO *et al.* 2007).

3) An apocytochrome *c* heme lyase

After its release from CcmC by the CcmAB ABC transporter, holoCcmE interacts with the heme lyase CcmFH complex, which catalyses the heme attachment reaction. Because heme must be in a reduced state prior to its ligation to apocytochrome *c*, its redox state must be controlled. Recent advances show that CcmF controls the redox state of heme (SAN FRANCISCO *et al.* 2011).

CcmF is a polytopic membrane protein with a conserved WWD signature motif and like CcmC, the protein belongs to the putative heme handling protein family (LEE *et al.* 2007). Spectroscopic and biochemical analysis of recombinant CcmF revealed the presence of a structural *b*-type heme (RICHARD-FOGAL *et al.* 2009; SAN FRANCISCO *et al.* 2011; SANDERS *et al.* 2008). Unlike *c*-type heme, which is linked covalently to the protein, *b*-type heme is coordinated via histidine ligands (FUFEZAN *et al.* 2008). Mutagenesis studies have identified two transmembrane histidines in CcmF as ligands of the structural *b*-heme (REN *et al.* 2002; SAN FRANCISCO *et al.* 2011). Based on *in vitro* experiments, the *b* heme of CcmF was shown to be reduced by quinones (KRANZ *et al.* 2009; RICHARD-FOGAL *et al.* 2009). Because holoCcmE was found to contain heme in the oxidized form, the proposed model is that CcmF accepts electrons from quinol to reduce the *b* heme, which subsequently reduces the heme in holoCcmE (RICHARD-FOGAL *et al.* 2009). It is not clear if the CcmF-dependent reduction of heme triggers its release from holoCcmE. Note that a direct experimental evidence that CcmF acts as a quinol-holoCcmE oxidoreductase is still lacking.

As part of its synthetase activity, CcmF is predicted to interact with holoCcmE and mobilize the heme from CcmE for attachment to the apocytochrome *c* substrate

(FEISSNER *et al.* 2006b; REN *et al.* 2002; RICHARD-FOGAL *et al.* 2009). Periplasmically located histidines, adjacent to the WWD domain were shown to act as ligands of the heme bound in holoCcmE (RICHARD-FOGAL and KRANZ 2010; RICHARD-FOGAL *et al.* 2009). It is likely that the WWD domain binds holoCcmE heme, precisely positioning the vinyl side chains to accept the C*XX*CH motif of the apocytochrome *c*. The synthetase reaction is believed to be spontaneous, as long as the iron of heme is reduced.

CcmF must also recognize the apocytochrome *c* C*XX*CH motif for the synthetase activity and CcmH has been postulated to fulfil this role based on its physical association with CcmF (MEYER *et al.* 2005; RAYAPURAM *et al.* 2008; REN *et al.* 2002; SANDERS *et al.* 2008; VERISSIMO *et al.* 2011). Stereospecificity is a key element in cytochrome *c* assembly. The vinyl-2 and -4 groups are always attached specifically to the N- and C-terminally located of Cys1 and Cys2 of the C*XX*CH heme binding motif of apocytochrome *c*. The mechanism underlying this ubiquitous specificity of heme-apocytochrome *c* ligation is unknown. The fact that a CcmH-apocytochrome c complex may be the direct target of CcmG reducing activity *in vivo*, suggests that CcmF associated with holoCcmE and CcmH may ensure the stereospecificity for the heme attachment reaction. Finally, it is important to note that the detailed mechanism of formation of the two thioether bonds at the CcmFH active site remains an open question in the field.

1.3.4.2. System I in mitochondria of plants

Based on the occurrence of Ccm-like proteins encoded in the nuclear and mitochondrial genomes of land plants, a System I assembly pathway was proposed to operate in plant

mitochondria (GIEGE *et al.* 2008; MEYER *et al.* 2005; RAYAPURAM *et al.* 2007; SPIELEWOY *et al.* 2001) (see Table 1.1). Several experimental investigations in *Arabidopsis* support the implication of the Ccm components in mitochondrial cytochrome *c* assembly. Plant System I displays notable differences from bacterial System I (GIEGE *et al.* 2008). While CcmABCEFH can be recognized on the basis to their sequence similarity to bacterial Ccm, CcmD, CcmI (corresponding to the C-terminal part of *E.coli* CcmH) and the prototypical components of the thio-reduction pathway (*e.g.* DsbD/CcdA and the thioredoxin-like CcmG) cannot be identified in land plant genomes. It is conceivable that CcmDIG and DsbD/CcdA cannot be recognized in plant genomes because their sequence is very divergent from their bacterial counterparts. It is also possible that their activity is not required for cytochrome *c* assembly in the mitochondrial IMS. While biochemical analyses of *Arabidopsis* CcmA (AtCcmA), CcmE (AtCcmE) and CcmH (AtCcmH) is compatible with a role for these components in cytochrome *c* maturation, there is still no direct genetic evidence for their function in cytochrome *c* and c_1 assembly, the only two *c*-type cytochromes involved in mitochondrial respiration.

AtCCMA localizes to the mitochondrial matrix and was shown to be associated with the inner membrane (RAYAPURAM *et al.* 2007). In agreement with its sequence similarity to bacterial CcmA, a recombinant form of AtCCMA exhibits ATPase activity (RAYAPURAM *et al.* 2007). Interaction of AtCCMA with AtCcmB, the membrane component of the ABC transporter, at the inner membrane is supported by yeast two-hybrid experiments (RAYAPURAM *et al.* 2007). ATP/Mg^{2+} was shown to facilitate AtCCMA dissociation from the mitochondrial membrane, most likely due to conformational changes induced by ATP

hydrolysis that affect the interaction with the transmembrane domains of AtCcmB (RAYAPURAM *et al.* 2007). Interestingly, AtCCMA occurs in a 480 kDa complex that does not contain other heme delivery proteins such as CCME or CcmF (see below) or CCMH (RAYAPURAM *et al.* 2007).

AtCCME is a peripheral protein of the mitochondrial inner membrane with its heme binding domain exposed to the mitochondrial IMS (SPIELEWOY *et al.* 2001). In *E. coli*, AtCCME has the ability to bind heme covalently via its conserved histidine when bacterial CcmC is co-expressed but cannot substitute for CcmE in cytochrome *c* maturation (SPIELEWOY *et al.* 2001). This was taken as an indication that the function of plant CCME as a heme chaperone is also conserved despite the fact that holoCCME could not be detected in mitochondria, presumably because it is a low abundance molecule.

While Ccm heme delivery components are conserved in plant mitochondria, this is not the case for the system I thioreduction pathway with the notable exception of AtCCMH. AtCCMH is an integral inner membrane protein with a domain containing a RC*XX*C motif facing the IMS (MEYER *et al.* 2005). Yeast two-hybrid experiment shows an interaction between the IMS-facing domain of AtCCMH and apocytochrome *c*. Moreover, *in vitro* redox assays with a recombinant form of AtCCMH show that the RC*XX*C motif can reduce a C*XX*CH disulfide in a model apocytochrome *c* peptide. Similarly to bacteria, AtCCMH was proposed to be involved in the reduction of the C*XX*CH heme binding motif of apocytochromes *c*. Interestingly, plant CcmF is encoded by a mitochondrial gene that has been split to multiple genes ($CcmF_{N1}$, $-F_{N2}$, $-F_C$), each of

which encodes an integral inner membrane protein with similarity to a corresponding domain of bacterial CcmF (GIEGE *et al.* 2008; RAYAPURAM *et al.* 2008). In *Arabidopsis* $CcmF_{N2}$ contains an IMS facing WWD domain that was shown to interact with both apoforms of cytochrome *c* and c_1 in a yeast two-hybrid assay (RAYAPURAM *et al.* 2008). The occurrence of $CcmF_{N1}$, $-F_{N2}$, $-F_C$ in a membrane complex supports the view that all three CcmF proteins constitute a functional heme lyase in plant mitochondria (RAYAPURAM *et al.* 2008). A role of $CcmF_C$ in *c*-type cytochrome maturation was recently evidenced through the identification of a splicing defect in an intron of the *Arabidopsis* $CcmF_C$ gene that results in decreased accumulation of holoform of cytochrome *c* and c_1 (FRANCS-SMALL *et al.* 2012). AtCCMH and $AtCcmF_{N2}$ co-localize in a complex and interact on the basis of yeast two-hybrid experiments, further underscoring the functional relationship between CcmF and CCMH (RAYAPURAM *et al.* 2008). It is likely that plant CcmF and CcmH form a complex and need to cooperate for the heme ligation reaction, similarly to the bacterial CcmFH synthase.

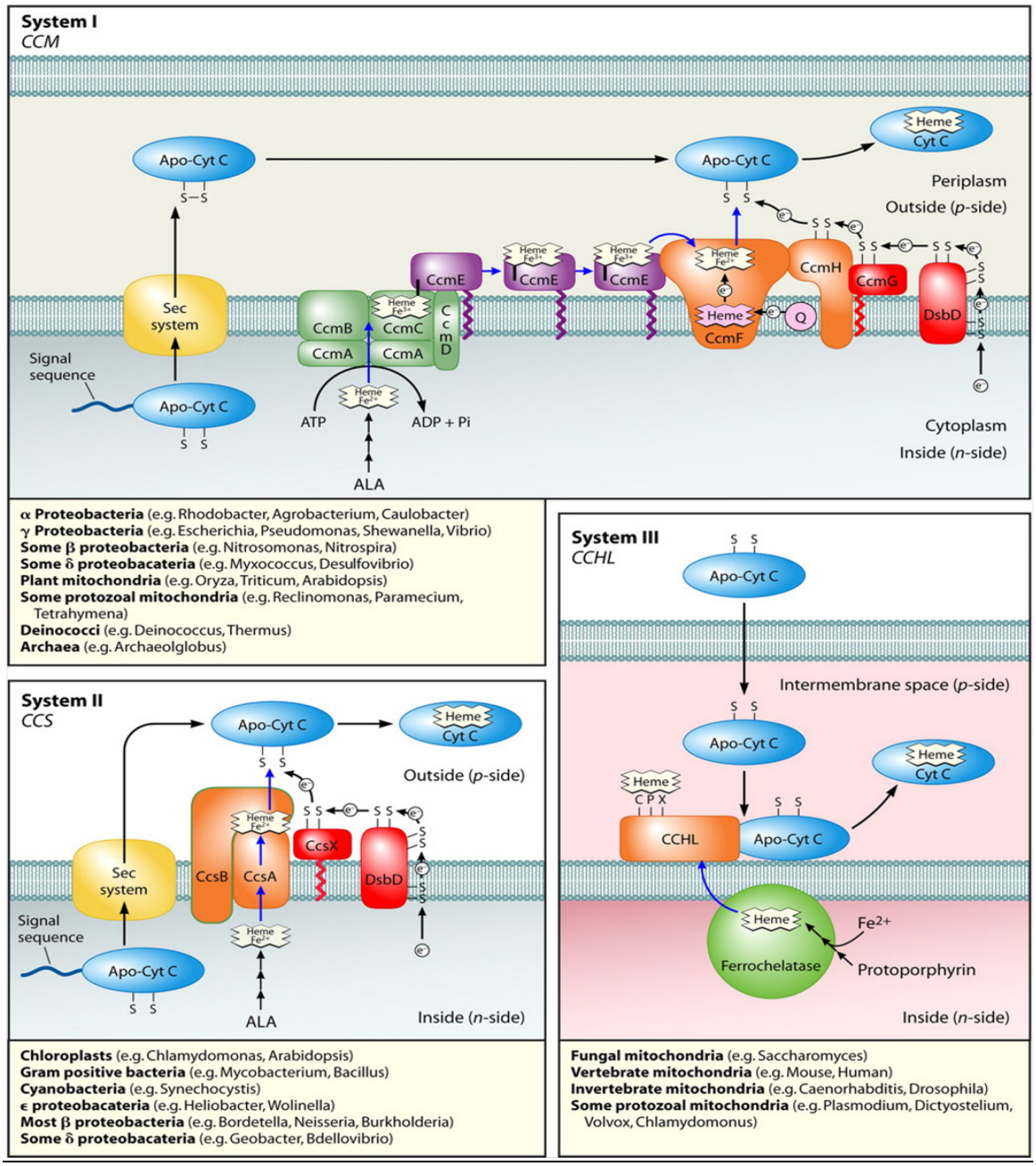


Figure 1.7. Working models for cytochrome *c* biogenesis by systems I, II, and III.

Models include trafficking and oxidation states of heme, as well as the pathways for apocytochrome reduction (in red for system I and system II). Representative organisms possessing each system are listed under the models. Source: (KRANZ *et al.* 2009). Representative organism for System I: *Escherichia coli*, System II: *Bordetella pertussis*, System III: *Saccharomyces cerevisiae*.

1.3.4.3. System II/CCS

System II also referred to as the CCS pathway ("CCS" for Cytochrome *C* Synthesis) first emerged through the genetic analyses of cytochrome *c* deficient mutants in the green alga *Chlamydomonas reinhardtii* (DREYFUSS *et al.* 2003; INOUE *et al.* 1997; XIE *et al.* 1998; XIE and MERCHANT 1996) and later in bacterial models such as *B. subtilis* (LE BRUN *et al.* 2000; SCHIÖTT *et al.* 1997a; SCHIÖTT *et al.* 1997b) and *Bordetella pertussis* (see Figure 1.7) (BECKETT *et al.* 2000; FEISSNER *et al.* 2005; KRANZ *et al.* 2002). The *ccs* mutants were initially identified through studies of plastid cytochrome *c* assembly in *Chlamydomonas* (KRANZ *et al.* 2009; NAKAMOTO *et al.* 2000; SANDERS *et al.* 2010; SIMON and HEDERSTEDT 2011). At least 7 CCS components, corresponding to the gene products of plastid *ccsA* and nuclear *CCS1* to *CCS6* (DREYFUSS and MERCHANT 1999; HOWE and MERCHANT 1992; HOWE *et al.* 1995; PAGE *et al.* 2004; XIE *et al.* 1998) have been implicated in the heme attachment reaction to the apoforms of plastid cytochromes *c*. Despite the pioneer discovery in *Chlamydomonas* of CcsA and Ccs1, the defining components of this system (INOUE *et al.* 1997; XIE and MERCHANT 1996), most of the progress in understanding system II is derived from experimental investigations in bacterial systems. Surprisingly, genetic screens reveal that only four assembly factors are required for cytochrome *c* biogenesis in bacterial system II (BECKETT *et al.* 2000; KRANZ *et al.* 2002; LE BRUN *et al.* 2000). These assembly factors are named CcsA, CcsB, CcsX and DsbD in *Bordetella pertussis* and ResC, ResB, ResA and CcdA in *B. subtilis*, respectively (see Table 1.1). Bacterial CcsA/ResC and CcsB/ResB are the orthologs of plastid CcsA and Ccs1, respectively. In the following paragraph, I will detail the

proposed function of CcsA/ResC and CcsB/ResB in cytochrome *c* assembly. Because this description is relevant to plastid and bacterial orthologs, the *p*-side and *n*-side terminology will be used to refer to lumen/periplasm and stroma/cytoplasm, respectively.

Plastid and bacterial CcsA/ResC are polytopic membrane proteins with a conserved WWD domain (like CcmC and CcmF) and three conserved histidine residues (two on the *p*-side flanking the WWD motif and one on the *n*-side) (AHUJA *et al.* 2009; HAMEL *et al.* 2003; KRANZ *et al.* 2009; LE BRUN *et al.* 2000). Tryptophan within the WWD domain and conserved histidine residues are required for CcsA/ResC activity in cytochrome *c* maturation (HAMEL *et al.* 2003). Because tryptophan and histidines are a common theme in heme handling proteins, CcsA/ResC was proposed to function in heme handling (HAMEL *et al.* 2003).

Plastid and bacterial Ccs1/CcsB are also polytopic membrane proteins with a large soluble C-terminal domain on the *p*-side, essential for their activity and proposed to function in chaperoning apocytochrome *c* (AHUJA *et al.* 2009; DREYFUSS *et al.* 2003; FEISSNER *et al.* 2005; KRANZ *et al.* 2009). A single histidine within the last transmembrane domain preceding the *p*-side C-terminal domain is also required for cytochrome *c* assembly (DREYFUSS *et al.* 2003; FRAWLEY and KRANZ 2009). The finding that plastid and bacterial CcsA are found in a complex with Ccs1/CcsB led to the proposal that these two proteins might function together in cytochrome *c* assembly (see Figure 1.7) (FEISSNER *et al.* 2005; HAMEL *et al.* 2003; XIE *et al.* 1998). This view is reinforced by the fact that Ccs1/CcsB and CcsA proteins are naturally fused into one large polypeptide (called CcsBA) in few bacteria such as *Helicobacter*, *Bacteroides* and

Wolinella. Overexpression of *Helicobacter* CcsBA in a *E.coli* strain deleted for the System I *CcmABCDEF* genes is necessary and sufficient for the assembly of a reporter holocytochrome *c* (FEISSNER *et al.* 2006b). Thus, CcsBA corresponds to the system II cytochrome *c* synthetase. Spectral analysis of the recombinant CcsBA protein indicates that the purified protein contains a *b* type heme that was shown to be in the reduced state (Fe^{2+}). Mutations of either of the two *p*-side histidines flanking the WWD motif changed the redox state of the *b*-heme from reduced to oxidized (Fe^{3+}), an indication that the heme group is on the *p*-side of CcsBA (FRAWLEY and KRANZ 2009). On this basis, the two *p*-side histidines were proposed to ligand the iron of the heme substrate in the reduced state for covalent thioether linkage. Mutation of the transmembrane *n*-side histidines located in the CcsA or CcsB domain of CcsBA decreases the amount of reduced *b*-heme on the *p*-side (FRAWLEY and KRANZ 2009). This led to the proposal that the transmembrane histidines define an entry site for channeling the heme substrate to the *p*-side where it is attached to apocytochrome *c*.

The working model is that CcsA and Ccs1/CcsB together form a transmembrane heme delivery system from cytosol to periplasm in bacteria or from stroma to thylakoid lumen in plastids (cytosol and stroma correspond to the *n*-side, periplasm and thylakoid lumen correspond to the *p*-side) for the heme ligation reaction (BECKETT *et al.* 2000; DREYFUSS *et al.* 2003; FEISSNER *et al.* 2006a; GOLDMAN *et al.* 1998; HAMEL *et al.* 2003). The proteins define two heme binding sites. The first one coordinates the reduced heme substrate localized at the channel entry formed by histidines in Ccs1/CcsB and CcsA on the *n*-side of the membrane. The second site, on the *p*-side, is composed of the WWD

domain and two flanking histidines of CcsA and protects the heme from oxidation. This heme binding domain is probably the active site of the cytochrome *c* synthase. Once the apocytochrome C*XX*CH motif is brought near the reduced heme tethered by the WWD domain of CcsA, the ligation reaction to form the thioether bond is believed to occur spontaneously.

The requirement for a thiol-reducing pathway in system II was established through classical and reverse genetic approaches primarily in bacterial systems but also in plastid through my work (see chapters 2 and 3). Components of this pathway include a thiol-disulfide transporter of the CcdA/DsbD family and ResA/CcsX, a membrane anchored periplasm-facing thioredoxin-like protein, which are required for the assembly of multiple bacterial cytochromes *c* (BARDISCHEWSKY and FRIEDRICH 2001; BECKETT *et al.* 2000; COLBERT *et al.* 2006; DESHMUKH *et al.* 2000; ERLENDSSON *et al.* 2003; ERLENDSSON and HEDERSTEDT 2002; LE BRUN *et al.* 2000; SCHIÖTT *et al.* 1997a). Bacterial mutants for CcdA/DsbD or ResA/CcsX are impaired in cytochrome *c* assembly and the ability of exogenous thiol compounds to by-pass the lack of these factors, *in vivo*, substantiates the view that the redox components of system II have a thio-reducing activity (BARDISCHEWSKY and FRIEDRICH 2001; BECKETT *et al.* 2000; ERLENDSSON and HEDERSTEDT 2002; FEISSNER *et al.* 2005). Erldensson *et al*. and other groups have proposed that CcdA/DsbD and ResA/CcsX constitute the redox relay required for the reduction of the sulfhydryls at the C*XX*CH heme binding site of apocytochrome *c* (ERLENDSSON *et al.* 2003; ERLENDSSON and HEDERSTEDT 2002). Interestingly, in some bacteria, orthologs of the thiol-disulfide transporter and the thioredoxin-like protein are

encoded by adjacent genes, giving support that they might act together in the same redox pathway (METHE *et al.* 2003; NAKAMURA *et al.* 2003). The working model of the thiol-reducing pathway in bacterial system II is as follows: reducing equivalents are transferred to DsbD/CcdA from a cytoplasmic thioredoxin that is maintained in a reduced state via the activity of the NADPH-dependent thioredoxin reductase. Electrons are conveyed via a thiol-disulfide exchange reaction through DsdD/CcdA to CcsX/ResA that maintains apocytochrome *c* sulfhydryls in a reduced state prior to heme ligation (see Figure 1.7). Experimental proof for this electron transfer *in vivo* is still lacking but detailed *in vitro* analysis of ResA supports its role as an apocytochrome *c* C*XX*CH disulfide reductase (COLBERT *et al.* 2006; CROW *et al.* 2004; ERLENDSSON *et al.* 2003). Note that similarly to System I, DsbD or CcdA also occurs in system II bacteria (see sections 1.2.1.5 and 1.2.2). Interestingly, loss of the thio-reducing pathway in System I and System II through mutation in the genes encoding the thiol-disulfide transporter CcdA/DsbD or the thioredoxin-like protein (CcmG in system I, ResA/CcsX in system II) can be compensated by inactivation of the thio-oxidizing pathway (*i.e*. mutations in the *dsbAB* or *BdbBC* genes, see sections 1.2.1 and 1.2.2) (DESHMUKH *et al.* 2000; ERLENDSSON and HEDERSTEDT 2002; TURKARSLAN *et al.* 2008). This supports the idea that the thio-reduction pathway in the context of cytochrome *c* assembly is only required to counteract the presence of the thio-oxidative pathway. In absence of the thio-reduction pathway, apocytochromes *c* become oxidized by the disulfide-bond forming enzymes whereas in absence of the thio-oxidation pathway, the thioreduction pathway is no longer required for cytochrome *c* assembly.

Pathway	System I	System II	System III
Saccharomyces cerevisiae	-	-	Mitochondria CCHL CC_1HL Cyc2p
Homo sapiens	-	-	Mitochondria HCCS
Arabidopsis thaliana	Mitochondria CCMA,-B,-C, -E, $-F_{N1}$, $-F_{N2}$, $-F_C$, -H[a]	Plastid [c]CcsA [d]CCS1 [e]HCF164 CCDA	-
Chlamydomonas reinhardtii	-	Plastid [c]CcsA, [d]CCS1 to 6 CCDA	Mitochondria CCHL CC_1HL
Escherichia coli γ proteobacteria	CcmA, -B, -C, -D, -E, -F, -G[b], -H[a], DsbD	-	-
Rhodobacter capsulatus α proteobacteria	CcmA, -B, -C, -D, -E, -F, -G[b], -H[a], -I[a], CcdA	-	-
Bacillus subtilis Gram-positive bacteria	-	[c]ResC [d]ResB [e]ResA CcdA	-
Bordetella pertussis *β* proteobacteria	-	[c]CcsA [d]CcsB [e]CcsX DsbD	-
Helicobacter hepaticus ε proteobacteria	-	CcsBA [e]CcsX DsbD	-

Table 1.1. The proteins involved in System I, System II and System III biogenesis pathways and sites of assembly are indicated.

[a]Rhodobacter and *Arabidopsis* CcmH correspond to the N-terminal domain of E. coli CcmH.
Rhodobacter Ccml is equivalent to the C-terminal domain of E. coli CcmH.
[b]CcmG corresponds to DsbE in some organisms.
[c]CcsA and ResC are orthologs.
[d]CcsB and ResB are orthologs of Ccs1.

continued

Table 1.1. continued.
[e]HCF164, ResA and CcsX are orthologs.
Ccm, cytochrome *c* maturation; Ccs, cytochrome *c* synthesis; Res, respiration; CcdA, cytochrome *c* defective; Dsb, disulfide bond; CCHL/CC1HL, cytochrome c/c_1 heme lyase; Cyc2, locus 2 controlling
cytochrome *c* assembly; HCCS, holo cytochrome *c* synthase; p and n, the positive and negative side of the energy transducing membrane, respectively; -, absent. Source: modified from (BONNARD *et al.* 2010).

1.3.4.4. System III/CCHL

System III is restricted to mitochondria and is required for the assembly of membrane-bound cytochrome c_1 and soluble cytochrome *c*, the two IMS resident *c*-type cytochromes that function in the respiratory electron chain (BONNARD *et al.* 2010). The defining component of this assembly pathway is CCHL, the prototypical cytochrome *c* heme lyase that localizes to the mitochondrial IMS (see Figure 1.7). A survey of sequenced genomes indicates that CCHL-like proteins seem to be exclusively found in fungi, animals and green algae (see Table 1.1) (ALLEN 2011; BERNARD *et al.* 2003; FERGUSON *et al.* 2008; GIEGE *et al.* 2008). System III in the mitochondria of unicellular eukaryotes is defined by CCHL (cytochrome *c* heme lyase) and CC_1HL (cytochrome c_1 heme lyase), two related proteins that were first identified through the genetic analysis of cytochrome *c* and c_1 deficients mutants in fungi (CERVERA *et al.* 1998; DRYGAS *et al.* 1989; DUMONT *et al.* 1987; NARGANG *et al.* 1988; ZOLLNER *et al.* 1992). System III in the mitochondria of multicellular eukaryotes, including humans, is characterized by the presence of a single heme lyase, called HCCS for holocytochrome *c* synthase, which exhibits both CCHL and CC_1HL activities (ALLEN 2011; BERNARD *et al.* 2003; PRAKASH *et al.* 2002; SCHAEFER *et al.* 1996; SCHWARZ and COX 2002; STEINER *et al.* 1996). The

heme lyase activity is inferred from the fact that CCHLs interact with heme via heme binding sites (CPX where X is V, M, I and L) (STEINER *et al.* 1996) and also with apoforms of cytochromes *c* (BERNARD *et al.* 2003; CORVEST *et al.* 2010; CORVEST *et al.* 2012; MAYER *et al.* 1995; NICHOLSON *et al.* 1989). However, deletion of the heme binding sites does not appear to be essential for cytochrome *c* assembly (Hamel, unpublished results) (STEINER *et al.* 1996; STEVENS *et al.* 2011) and the biochemical activity of the CCHLs remains unclear.

In contrast to systems I and II, exhaustive genetic screens failed to uncover system III components involved in the transport of heme (from its site of synthesis in the mitochondrial matrix to the IMS) or delivery of reductants for maintenance of the C*XX*CH sulfhydryls (see Figure 1.7) (SHERMAN 1990). One notable exception is Cyc2p, an inner membrane anchored, IMS-facing flavoprotein discovered in yeast (BERNARD *et al.* 2003; BERNARD *et al.* 2005; CORVEST *et al.* 2010; DUMONT *et al.* 1993; PEARCE *et al.* 1998). The finding that Cyc2p is able to catalyze the NAD(P)H-dependent reduction of heme *in vitro* was taken as an indication that the protein's role may be to control the redox state of the iron in the heme attachment reaction to apocytochromes *c*, possibly in a complex with CCHL (CORVEST *et al.* 2012). Other mechanisms for heme reduction in system III mitochondria must exist based on the fact that Cyc2p appears to be restricted to fungi (ALLEN 2011).

1.4. Redox pathways in chloroplast

1.4.1. The function of chloroplast in photosynthesis

The chloroplast is an organelle defining three compartments, the intermembrane space, the stroma and the thylakoid lumen, which are delimited by three membranes, the outer envelope membrane, the inner envelope membrane and the thylakoid membrane (see Figure 1.9) (GUTENSOHN *et al.* 2006). According to the endosymbiotic theory, chloroplasts are derived from a primary endosymbiotic event between a cyanobacterium and a eukaryotic organism (DOUZERY *et al.* 2004; YOON *et al.* 2004), followed by a massive transfer of genes from the symbiont to the host nucleus (MARTIN *et al.* 2002). Accordingly, 98% of all chloroplast proteins must be imported after translation from the cytosol (HORMANN *et al.* 2007). The small number of proteins encoded by the plastid genome mainly resides in the thylakoids and contributes to key functions of the chloroplast.

Photosynthesis comprises two distinct phases that occur entirely within the chloroplast. The light-dependent reactions of oxygenic photosynthesis (*i.e.* producing oxygen by opposition to anoxygenic photosynthesis that does not generate oxygen in some bacteria) take place in the thylakoid membrane and are catalyzed by several complexes that include photosystem II (PSII), photosystem I (PSI), their associated light-harvesting systems, the cytochrome b_6f complex and the ATP synthase. Each of these complexes consists of multiple subunits, chlorophyll pigments and redox cofactors. Upon light absorption by the antennae, the excitation energy is channeled to the reaction centers to

oxidize the chlorophyll dimers P680 and P700 in PSII and PSI, respectively (see Figure 1.8).

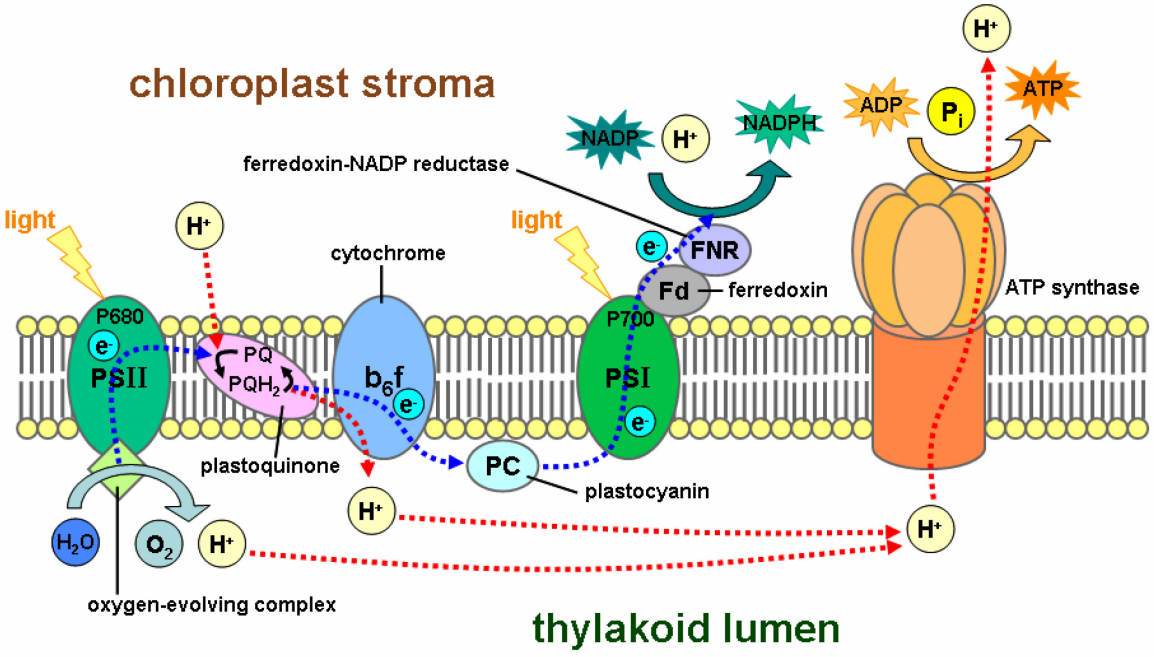


Figure 1.8. Light-dependent reactions of photosynthesis at the thylakoid membrane.

The electron-transport system consists of three proteins complexes: PSII, the cytochrome b_6f complex and PSI which are involved in electron transport and connected by electron carriers plastoquinone (PQ) and plastocyanine (PC). Light-driven transport of electrons from H_2O to $NADP^+$ is coupled with the transport of protons into the thylakoid lumen. Additional protons are split off from water by the oxygen evolving complex, yielding O_2. The resulting proton gradient powers the synthesis of ATP by the ATP synthase. The membrane also contains light-harvesting complexes (not shown) whose component pigments transfer their excitations to PSI and PSII. Fd represents ferredoxin and FNR represents Ferredoxin-NADP reductase.

The electron donor side of PSII acts as a strong oxidant and is capable of oxidizing water, which besides yielding molecular oxygen and protons in the thylakoid lumen, is the source of electrons for the photosynthetic electron transport chain. The electrons, originating from P680 excitation, are transferred across the thylakoid membrane to the plastoquinone pool, which transfers its electrons to the cytochrome b_6f complex. After a

series of complex reactions, some electrons are transferred from the cytochrome b_6f *via* soluble plastocyanin to PSI while other electrons are routed within the cytochrome b_6f and participate in the proton pumping activity of this complex. Upon charge separation within PSI, the electrons are transferred from plastocyanin to ferredoxin and finally to NADP. Photosynthetic electron transfer generates a proton gradient across the thylakoid membrane that drives the production of ATP *via* the ATP synthase (see Figure 1.8).

The chemical energy (ATP) and the reductant (NADPH) are then used in the light-independent reactions that take place in the chloroplast stroma, in which CO_2 is fixed by Rubisco to produce sugars. Subsequently, this carbohydrate is either immediately exported to the cytosol or is stored within the chloroplast as starch. Beyond photosynthesis, the chloroplast is also the site of fatty acid biosynthesis, nitrate assimilation and amino-acid biosynthesis.

1.4.2. Thiol-disulfide based chemistry in the chloroplast

Many studies have provided extensive information about the role of catalyzed thiol-disulfide chemistry in regulating the activities of several proteins in the stroma but little information is available on such a process in the intermembrane space or the thylakoid lumen (see Figure 1.9).

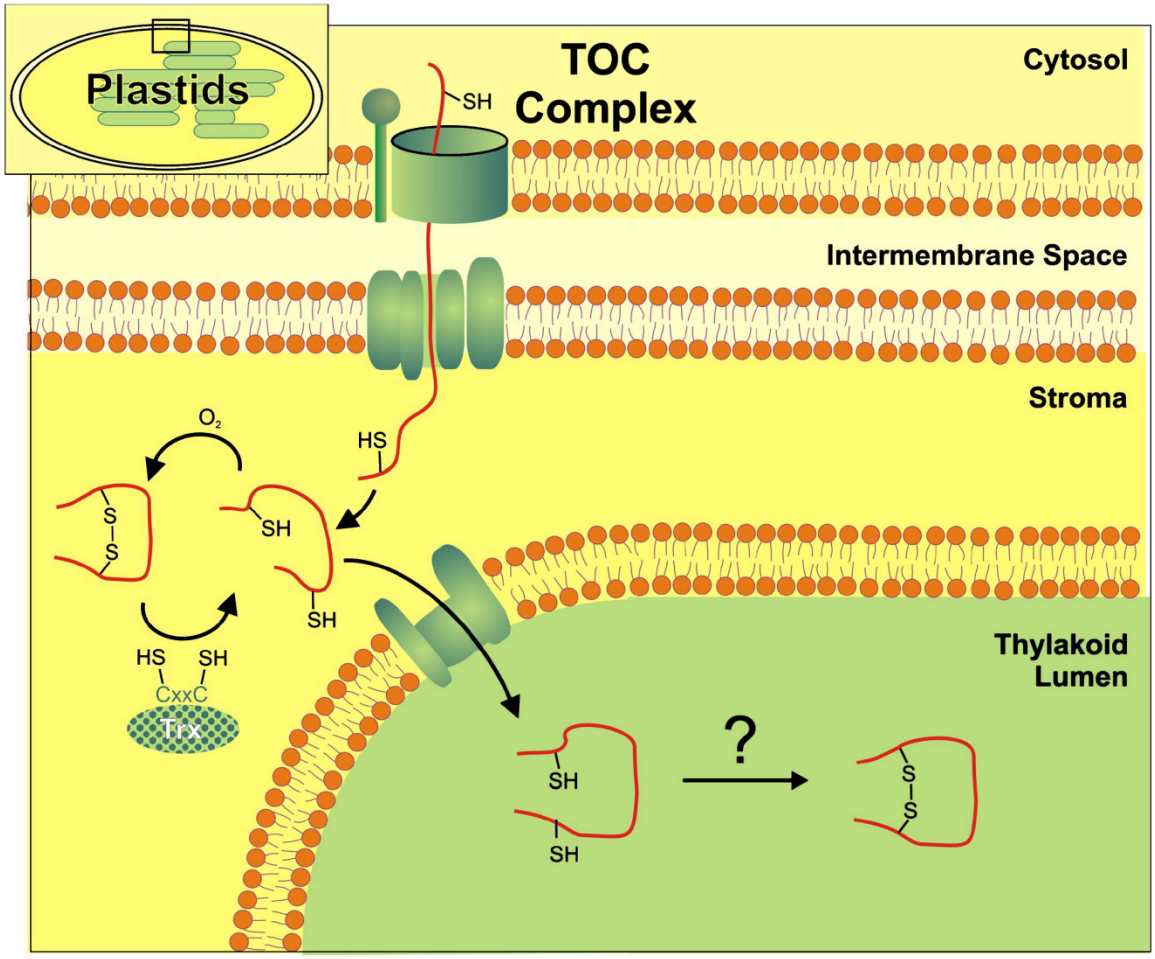


Figure 1.9. Protein oxidation in plastids.

Redox regulation is poorly understood in plastids. Some proteins of the thylakoid lumen are oxidized after their import from the stroma but the components that mediate protein oxidation are unknown. In the stroma, proteins are maintained in a reduced state by thioredoxins. Source: (HERRMANN *et al.* 2009).

My research project is a contribution to the study of catalyzed thiol-disulfide chemistry in the chloroplast with particular focus on the thio-reducing pathway operating in the thylakoid lumen compartment. This process was studied in the context of plastid cytochrome *c* assembly.

1.4.2.1. Thiol-disulfide based chemistry in the stroma

A large number of stroma-localized proteins are regulated by the ferredoxin/thioredoxin system via reversible oxidation and reduction of cysteine residues (BUCHANAN and BALMER 2005; DIETZ 2003; DIETZ and SCHEIBE 2004). This system composed of ferredoxin (Fd), ferredoxin-thioredoxin reductase (FTR) and thioredoxin (Trx), targets soluble and membrane bound proteins by fine regulation of their redox states (see Figures 1.9 and 4.2). In the light, thioredoxins act by reducing disulfide bonds in stroma-localized targets. In the dark, the reversible reaction is the oxygen-dependent oxidation of the sulfhydryls in the protein targets (see Figure 1.9). The thioredoxin-dependent reduction of disulfides activates the targets while the oxygen-dependent oxidation of the sulfhydryls has the opposite effect. In plant chloroplasts, ferredoxin, a potent electron donor, is directly reduced by the photosynthetic electron transport chain at the acceptor site of PSI on the stroma (see Figure 1.8). Ferredoxin is an iron sulfur-based molecule, which distributes electrons to various acceptors, such as $NADP^+$ mediated by FNR and stromal thioredoxins catalyzed by FTR.

Stromal thioredoxins (Trx *f*, Trx *m*) were first identified as essential factors in light-mediated activation of Calvin cycle enzymes. For instance, Trx *f* activates fructose-1,6-bisphosphatase, sedoheptulose-1.7-bisphosphatase and phosphoribulokinase that are part of the Calvin cycle. The ATP synthase, another protein involved in photosynthesis (see Figure 1.8) is also regulated by interaction with Trx *f* (DIETZ 2003). This thioredoxin-mediated regulation is not only restricted to metabolic enzymes but also affects components such as TIC55, PTC52 and PAO (BARTSCH *et al.* 2008). TIC55 is part of the

general import machinery responsible for the transport of the small subunit of RubisCO (involved in CO_2 fixation) and other photosynthetic precursors into the chloroplast. PTC52 establishes a distinctive translocon involved in the transport of the precursor NADPH:protochlorophyllide (Pchlide) oxidoreductase A (pPORA). POA is a pheophorbide *a* oxygenase involved in chlorophyll breakdown in senescent chloroplasts. These three proteins share C*XX*C motifs and are localized in the inner membrane of chloroplasts. They appear to be more active in their reduced forms than their oxidized forms (BARTSCH *et al.* 2008; BUCHANAN and BALMER 2005; MONTRICHARD *et al.* 2009). Glutamine synthase and the small subunit of Rubisco are other examples of stromal enzymes targeted by thioredoxins (MOTOHASHI *et al.* 2001).

1.4.2.2. Thiol-disulfide based chemistry in the thylakoid lumen

There is now mounting evidence that lumen-localized proteins require oxidized cysteines for their structure and/or activity (BUCHANAN and LUAN 2005; KARAMOKO *et al.* 2011; MONTRICHARD *et al.* 2009). Interestingly, several lumen-resident enzymes display thiol-dependent activities, an indication that thiol-disulfide chemistry (*i.e.* thio-oxidation and thio-reduction) might be a regulated process in this compartment. *At*FKBP13, a peptidyl-prolyl *cis-trans*-isomerase (PPIase) is active when a pair of cysteines is oxidized but inactive when reduced (see Figure 1.9) (BUCHANAN and LUAN 2005; GOPALAN *et al.* 2004; GUPTA *et al.* 2002b). STT7/STN7 is a kinase involved in adaptation to changes in light intensity. STT7 contains a transmembrane region that separates its catalytic kinase domain on the stromal side from its N-terminal end facing the thylakoid lumen with two

conserved cysteines. The fact that mutation of either conserved cysteine inactivates the enzyme suggests that the protein is active when the cysteines are disulfide bonded (DEPÈGE *et al.* 2003; LEMEILLE *et al.* 2009). Violaxanthin de-epoxidase (VDE) is an enzyme involved in dissipating excess light (SOKOLOVE and MARSHO 1976; YAMAMOTO and KAMITE 1972) and inhibited by DTT, suggesting that VDE is active in its oxidized form (NEUBAUER 1993). In addition, some structural subunits of the photosynthetic chain such as PsbO in PSII (BETTS *et al.* 1996; BURNAP *et al.* 1994; WYMAN and YOCUM 2005) and the Rieske in the b_6f complex (GOPALAN *et al.* 2004; GUPTA *et al.* 2002b) are known to be disulfide bonded.

The operation of one or several thio-oxidizing pathways in the thylakoid lumen is also supported by the finding that bacterial alkaline phosphatase (PhoA), an enzyme only active in its oxidized form (two disulfide bonds), is active when targeted to this compartment in tobacco (BALLY *et al.* 2008; SONE *et al.* 1997). A novel class of disulfide bond forming enzymes with similarity to VKOR was recently recognized in cyanobacteria, the presumed ancestor of chloroplasts, (SINGH *et al.* 2008) and in all photosynthetic eukaryotes (GROSSMAN *et al.* 2011). The disulfide bonding activity of cyanobacterial VKOR-like has been demonstrated through functional complementation of the bacterial *dsbAB* mutants (SINGH *et al.* 2008) and reconstitution of disulfide bond formation with the purified enzyme (LI *et al.* 2010b). However it is not clear if the cyanobacterial enzyme functions in the perisplasm and also in the thylakoid lumen because its cellular sublocalization has not been examined (SINGH *et al.* 2008). A VKOR-like protein is detected at the thylakoid membrane in *Arabidopsis* chloroplasts (ZYBAILOV

et al. 2008), and parallel investigation in our laboratory has established that this protein defines a thio-oxidizing pathway, operating in the thylakoid lumen (see chapter 4) (KARAMOKO *et al.* 2011).

By analogy to bacteria (see section 1.3.4), it is reasonable to expect that thio-reducing pathways also operate in the thylakoid lumen. Because *c*-type cytochromes also reside in the lumen, a thio-reduction pathway is probably required for covalent attachment of the heme cofactor to the C*XX*CH motif.

1.4.2.3. Plastid *c*-type cytochromes

The thylakoid lumen of plastids is known to house three *c*-type cytochromes, the membrane-bound cytochrome *f* and the soluble cytochromes c_6 and c_{6A}. Only cytochrome *f* and c_6 function as electron carriers in photosynthesis, cytochrome c_{6A} was discovered a decade ago and does not appear to function in photosynthesis. All plastid cytochromes *c* contain a single heme attached to a C*XX*CH motif on the apoprotein.

Cytochrome *f*, a catalytic subunit of the cytochrome b_6f complex is universal in all photosynthetic eukaryotes (and cyanobacteria) and is essential for photosynthesis (MARTINEZ *et al.* 1994). Cytochrome c_6 is exclusively found in the plastid of eukaryotic algae, where it is widely distributed among green, red and brown algal lineages (KERFELD and KROGMANN 1998; SANDMANN *et al.* 1983). Cytochrome c_6 is involved in the transfer of electrons from cytochrome *f* of the cytochrome b_6f complex to Photosystem I (see Figure 1.8) (MERCHANT and DREYFUSS 1998). In green algae and

cyanobacteria, cytochrome c_6 acts as a substitute for plastocyanin in Cu-deficient conditions (MERCHANT and BOGORAD 1987a; MERCHANT and BOGORAD 1987b).

A novel *c*-type cytochrome, cytochrome c_{6A} has been recently recognized in the thylakoid lumen of land plants and green algae but appears absent in other photosynthetic eukaryotes such as red algae and diatoms (WASTL *et al.* 2004). Cytochrome c_{6A} was discovered in *Arabidopsis* as a protein interacting with the lumen-localized immunophilin FKBP13 in a yeast two-hybrid screen (BUCHANAN and LUAN 2005; GUPTA *et al.* 2002a; GUPTA *et al.* 2002b). It was initially postulated that cytochrome c_{6A} acts as substitute for plastocyanin (GUPTA *et al.* 2002a), similarly to green algae where cytochrome c_6 can replace plastocyanin (MERCHANT and BOGORAD 1987a; MERCHANT and BOGORAD 1987b). However, loss of cytochrome c_{6A} in *Arabidopsis* has no visible phenotype even under Cu deficient conditions (HOWE *et al.* 2006). Moreover, an *Arabidopsis* plastocyanin-deficient mutant is unable to grow photoautotrophically even when cytochrome c_{6A} is overexpressed (MOLINA-HEREDIA *et al.* 2003; WEIGEL *et al.* 2003). Remarkably, cytochrome c_{6A} contains an additional C*XX*C (in addition to the typical C*XX*CH heme binding site) that is absent in algal and cyanobacterial cytochromes c_6 (HOWE *et al.* 2006). This motif is oxidized in the 3D structure of the molecule and a role for cytochrome c_{6A} as a redox sensor via this motif has been postulated (MARCAIDA *et al.* 2006). Cytochrome c_{6A} does not appear to function in the known electron transfer reactions of photosynthesis and this is compatible with its extremely low abundance in the thylakoid. Its function in the thylakoid lumen remains unknown (MARCAIDA *et al.* 2006).

1.4.2.4. A trans-thylakoid disulfide-reducing pathway required for photosynthesis

By analogy to the thio-reducing pathway of the bacterial system II, composed of CcdA/DsbD (the thiol disulfide transporter) and ResA (the thioredoxin-like), a similar system has been proposed for maintaining the cysteine thiols of apocytochromes *c* in a reduced state in the thylakoid lumen of plastids, the compartment topologically equivalent to the bacterial periplasm. CCDA and HCF164 (High Chlorophyll Fluorescence), first described in *Arabidopsis* are the best described plant orthologs of bacterial CcdA and ResA/CcsX proteins (LENNARTZ *et al.* 2001; PAGE *et al.* 2004). CCDA is a polytopic thylakoid membrane protein with six transmembrane domains (TMD) and two conserved cysteines in TMD1 and TMD4, similar to bacterial CcdA (MOTOHASHI and HISABORI 2010; PAGE *et al.* 2004). HCF164 is a membrane anchored protein with a typical WC*XX*C thioredoxin-like motif exposed to the thylakoid lumen, which displays some similarity to bacterial CcmG in System I and CcsX/ResA in System II (LENNARTZ *et al.* 2001; MOTOHASHI and HISABORI 2006). *Arabidopsis* mutants in the *CCDA* or *HCF164* genes are deficient for photosynthesis due to a defect in the cytochrome b_6f complex accumulation, which was attributed to loss of cytochrome *f* assembly. The cytochrome *f* deficient phenotype in *ccda* and *hcf164* mutants was believed to be caused by a defect in the heme attachment to apocytochrome *f* (LENNARTZ *et al.* 2001; PAGE *et al.* 2004). The implication of CCDA and HCF164 in a trans-thylakoid disulfide-reducing pathway for cytochrome *c* assembly is inferred from their sequence similarity to their bacterial counterparts and the finding that recombinant HCF164 exhibits a disulfide reductase activity (LENNARTZ *et al.* 2001; PAGE *et al.* 2004).

However, the activity of CCDA and HCF164 in cytochrome *c* assembly could not be ascertained because the heme attachment reaction was not monitored in the *ccda* and *hcf164* mutants (LENNARTZ *et al.* 2001; PAGE *et al.* 2004). There is also no *in vivo* experimental evidence for their reducing activity in the assembly of the b_6f complex.

1.4.3. Plastid cytochrome *c* assembly

1.4.3.1. *ccs* mutants

The *Chlamydomonas ccs* mutants (*ccs* for cytochrome *c* synthesis) were isolated on the basis of a dual deficiency in the holoforms of both cytochrome *f* and cytochrome c_6, the two lumen resident *c*-type cytochromes that function in photosynthesis (DREYFUSS and MERCHANT 1999; HOWE and MERCHANT 1992; INOUE *et al.* 1997; PAGE *et al.* 2004; XIE *et al.* 1998). Note that cytochrome c_6 is only expressed in Cu-deficient conditions as a replacement of plastocyanin, the electron carrier between the b_6f complex and PSI (MERCHANT and BOGORAD 1987a; MERCHANT and BOGORAD 1987b). All *ccs* mutants are photosynthetic deficient because loss of cytochrome *f* assembly results in a b_6f-minus phenotype (DREYFUSS and MERCHANT 1999; HOWE and MERCHANT 1992; INOUE *et al.* 1997; PAGE *et al.* 2004; XIE *et al.* 1998). Because apocytochrome *f* is plastid-encoded and apocytochrome c_6 is nuclear-encoded, the dual cytochrome f/c_6 deficiency was likely to be caused by a block at a step that is common to the biogenesis of both molecules. Indeed, pulse-chased experiments reveal that both plastid apocytochromes *c* are synthesized, imported in the thylakoid lumen, processed by lumen-resident signal peptidase but fail to be converted to their respective holoforms (HOWE and MERCHANT

1993; HOWE and MERCHANT 1994; XIE *et al.* 1998). Based on these experiments, it was concluded that the *ccs* mutants exhibit a defect in the heme attachment to apoforms of cytochrome *c* in the thylakoid lumen (HOWE and MERCHANT 1993; HOWE and MERCHANT 1994; XIE *et al.* 1998). The *ccs* mutants are also expected to display a defect in cytochrome c_{6A}. However, this could not be tested because the corresponding holoform could not be detected by heme stain (unpublished). The defect in the *ccs* strains is specific to plastid *c*-type cytochromes because plastocyanin, another metalloprotein in the lumen is normally assembled (HOWE and MERCHANT 1993; HOWE and MERCHANT 1994; XIE *et al.* 1998). Several independent mutagenesis experiments yielded a collection of *ccs* mutants that defined 7 loci, plastid *ccsA* and nuclear *CCS1* to *CCS6* (DREYFUSS *et al.* 2003; DREYFUSS and MERCHANT 1999; HAMEL *et al.* 2003; INOUE *et al.* 1997; PAGE *et al.* 2004; XIE *et al.* 1998; XIE and MERCHANT 1996).

1.4.3.2. Exogenous thiol rescue of the *ccs* mutants

In bacteria, loss of System II cytochrome *c* assembly factors with a proposed reducing activity (CcdA/DsbD, CcmG, CcsX and ResA), can be by-passed by provision of exogenous reduced thiols in the medium (BARDISCHEWSKY and FRIEDRICH 2001; BECKETT *et al.* 2000; DESHMUKH *et al.* 2000; ERLENDSSON and HEDERSTEDT 2002; FEISSNER *et al.* 2005). The current thinking is that the thiols act as a substitute for the reducing activity of the assembly factors and are able to chemically reduce a disulfide in the apocytochrome *c* C*XX*CH motif, a required step prior to the heme ligation reaction. In order to deduce a reducing activity for the gene product(s) of uncharacterized *CCS* loci,

we tested the ability of exogenous thiols (DTT, MESNA, cysteine) to rescue the photosynthetic growth of all *ccs* mutants. Surprisingly, application of exogenous thiols could rescue the photosynthetic deficiency of *ccs4* and *ccs5* mutants. Hence a disulfide-reducing activity was inferred for the *CCS4* and *CCS5* gene products.

Chapter 2 and chapter 3 document this exogenous thiol-dependent rescue, describe the molecular characterization of the *CCS5* and *CCS4* genes and define the activity of the corresponding gene products in a trans-thylakoid disulfide-reducing pathway.

CHAPTER 2

CCS5, A THIOREDOXIN-LIKE PROTEIN INVOLVED IN THE ASSEMBLY OF PLASTID C-TYPE CYTOCHROMES

2.1. Abstract

The *c*-type cytochromes are metalloproteins with a heme molecule covalently linked to the sulfhydryls of a C*XX*CH heme binding site. In plastids, at least six assembly factors (CCS) are required for heme attachment to the apoforms of cytochrome *f* and cytochrome c_6, in the thylakoid lumen. *CCS5*, controlling plastid cytochrome *c* assembly, was identified through insertional mutagenesis in the unicellular green alga *Chlamydomonas reinhardtii*. The complementing gene encodes a protein with similarity to *Arabidopsis thaliana* HCF164, which is a thylakoid membrane anchored protein with a lumen facing thioredoxin-like domain. HCF164 is required for cytochrome b_6f biogenesis but its activity and site of action in the assembly process has so far remained undeciphered. We demonstrate that CCS5 is a component of a trans-thylakoid redox pathway and operates by reducing the C*XX*CH heme binding site of apocytochrome *c* prior to the heme ligation reaction. We also show that CCS5 is the functional homolog of *Arabidopsis* HCF164. The proposal is based on the finding that 1) the *ccs5* mutant is rescued by exogenous thiols, 2) CCS5 interacts with apocytochrome *f*, c_6 and c_{6A} in a yeast two-hybrid assay, 3)

recombinant CCS5 is able to reduce a disulfide in the C*XX*CH heme binding site of apocytochrome *f* and 4) Expression of *Arabidopsis thaliana HCF164* in the chloroplast genome of *Chlamydomonas ccs5* mutant restores *c*-type cytochromes assembly.

2.2. Introduction

The *c*-type cytochromes are functionally versatile hemoproteins housed in energy-transducing membranes of bacteria, mitochondria and plastids where they participate in electron transfer reactions (THÖNY-MEYER 1997). Conversion of apo to holocytochromes *c* requires the covalent and stereospecific attachment of the heme cofactor to a C*XX*CH motif on the apoprotein, a process that was discovered to take place *via* at least three distinct assembly mechanisms, the so-called Systems I, II and III (BONNARD *et al.* 2010; FERGUSON *et al.*; GIEGE *et al.* 2008; HAMEL *et al.* 2009; KRANZ *et al.* 2009; SANDERS *et al.* 2010). System IV is a maturation pathway required for the covalent attachment of heme C_i to cytochrome b_6, in the b_6f complex of cyanobacteria and photosynthetic eukaryotes (KURAS *et al.* 1997; KURAS *et al.* 2007; LYSKA *et al.* 2007). This pathway is not a cytochrome *c* maturation system because cytochrome b_6 is very distinct from a typical cytochrome *c*.

The definition of the Systems I, II and III is based on the occurrence of prototypical assembly components that are the hallmark of each maturation pathway (FERGUSON *et al.*; HAMEL *et al.* 2009; KRANZ *et al.* 2009). In all three Systems (I, II, III), covalent attachment of the heme moiety occurs on the positive side of energy-transducing membranes (*i.e.* bacterial periplasm, mitochondrial intermembrane space, thylakoid

lumen) and requires minimally: 1) synthesis and transport across at least one biological membrane of apocytochromes *c* and heme, 2) reduction and maintenance of the heme ferrous iron and the C*XX*CH sulfhydryls prior to 3) formation of thioether bonds between the heme and apocytochromes *c* (FERGUSON *et al.*; GIEGE *et al.* 2008; HAMEL *et al.* 2009; KRANZ *et al.* 2009).

System II, the assembly pathway investigated in this paper, was originally discovered in the green alga *Chlamydomonas reinhardtii* through genetic studies of *ccs* mutants (*ccs* for *c*ytochrome *c s*ynthesis) that display a specific defect in membrane-bound cytochrome *f* and soluble cytochrome c_6, two thylakoid lumen resident *c*-type cytochromes functioning in photosynthesis (HOWE and MERCHANT 1992). All the *ccs* mutants exhibit a photosynthetic deficiency and fail to accumulate holoforms of both cytochrome *f* and c_6 because the heme attachment reaction, which is the only common step in the biogenesis of both proteins, is compromised (HOWE and MERCHANT 1992; HOWE *et al.* 1995; XIE *et al.* 1998). At least 7 loci, plastid *ccsA* and nuclear *CCS1* to *CCS6* were uncovered through genetic analysis and it is likely that saturating mutagenesis screens will reveal additional loci controlling the assembly process (DREYFUSS and MERCHANT 1999; HOWE and MERCHANT 1992; PAGE *et al.* 2004; XIE *et al.* 1998). CcsA and Ccs1, the defining components of this pathway are polytopic membrane proteins that were postulated to function together as a heme relay unit from stroma to lumen (DREYFUSS *et al.* 2003; GOLDMAN *et al.* 1998; HAMEL *et al.* 2003). Recently the *Helicobacter pylori* CcsBA protein, a naturally occurring fusion of the Ccs1 and CcsA orthologs, was proposed to work as a heme channel that also exhibits the heme ligation activity (FEISSNER *et al.*

2006b; FRAWLEY and KRANZ 2009). The involvement of redox chemistry in System II was first established *via* genetics in bacterial models. Components of this pathway include a thiol/disulfide membrane transporter of the CcdA/DsbD family (KIMBALL *et al.* 2003; PORAT *et al.* 2004; STIRNIMANN *et al.* 2006) whose implication in cytochrome *c* maturation was first demonstrated in the bacteria *Bacillus subtilis* and *Bordetella pertussis* (BARDISCHEWSKY and FRIEDRICH 2001; DESHMUKH *et al.* 2000; ERLENDSSON and HEDERSTEDT 2002). Another redox factor of the System II thiol reduction pathway is the membrane anchored periplasm-facing thioredoxin-like ResA/CcsX required for the assembly of multiple bacterial cytochromes *c* (BECKETT *et al.* 2000; ERLENDSSON *et al.* 2003; LENNARTZ *et al.* 2001). The placement of CcdA/DsbD and ResA/CcsX in a thiol reduction pathway is inferred from the finding that *ccda*/*dsbD* and *resA*/*ccsx* mutants can be rescued by exogenous thiols (BARDISCHEWSKY and FRIEDRICH 2001; BECKETT *et al.* 2000; DESHMUKH *et al.* 2000; ERLENDSSON *et al.* 2003; ERLENDSSON and HEDERSTEDT 2002; FEISSNER *et al.* 2005). The current thinking is that exogenous thiols substitute for the reducing activity of the redox factors and maintain the apocytochrome *c* C*XX*CH motif in a reduced form (*i.e.* sulfhydryls). The proposed model is that electrons are transferred from NADPH in the cytoplasm to the apocytochrome *c* C*XX*CH target in the periplasmic space through disulfide-dithiol exchange reactions involving sequentially a cytosolic NADPH-dependent thioredoxin reductase, a thioredoxin, CcdA/DsbD and ResA/CcsX (MÖLLER and HEDERSTEDT 2006; ORTENBERG and BECKWITH 2003). This view is supported by the finding that a recombinant form of ResA is active in thiol-disulfide exchange in an *in vitro* assay (ERLENDSSON *et al.* 2003).

By analogy to the bacterial membrane, provision of reducing equivalents to the thylakoid lumen is also expected in the context of plastid cytochrome *c* assembly. The occurrence of CcdA orthologs encoded in the plastid genomes of some photosynthetic eukaryotes suggests that plastids might have inherited a bacterial-like thiol reduction pathway (NAKAMOTO *et al.* 2000; PAGE *et al.* 2004). In the plant *Arabidopsis thaliana*, the CCDA protein localizes at the thylakoid membrane and loss-of-function *ccdA* mutations result in a photosynthetic deficient phenotype due to a defect in cytochrome b_6f complex assembly (MOTOHASHI and HISABORI 2010; PAGE *et al.* 2004). A possible redox partner for CCDA is the thylakoid membrane-bound lumen-facing HCF164 thioredoxin-like protein. This is suggested by the fact that loss of HCF164 function in *Arabidopsis* also leads to a cytochrome b_6f deficiency (LENNARTZ *et al.* 2001). If experimental investigations in *Arabidopsis* support the implication of the thiol-disulfide transporter CCDA and the thioredoxin-like HCF164 in the biogenesis of the cytochrome b_6f complex, they do not elucidate whether heme attachment to apocytochrome *f* is compromised by *ccda* or *hcf164* mutations because the heme attachment reaction has not been directly monitored in the vascular plant system (LENNARTZ *et al.* 2001; PAGE *et al.* 2004). However, in *Chlamydomonas*, cytochrome *f* and cytochrome c_6 are synthesized in different compartments (plastid vs. cytosol) and the only common step in biogenesis is the heme attachment. Therefore, *ccs* mutants that are deficient in both proteins are most likely defective at heme attachment. Indeed, pulse-chase analyses in the *ccs* mutants revealed the synthesis of apoform but not holoform of cytochrome *f* and c_6, indicative of a block in conversion (HOWE and MERCHANT 1992; HOWE *et al.* 1995; XIE *et al.* 1998).

The fact that the *Chlamydomonas CCS3* and *CCS4* loci are only defined by a single allele suggests that mutagenesis screens for plastid cytochrome *c* deficient mutants are not saturated (XIE *et al.* 1998). To identify additional *CCS* loci controlling plastid *c*-type cytochrome assembly, an insertional mutagenesis with the *ARG7Φ* marker (GUMPEL *et al.* 1994) was performed and led to the isolation of 80 photosynthesis-minus mutants that displayed a fluorescence kinetic characteristic of a deficiency in the cytochrome b_6f complex (see Figures 2.2C and 2.5A for example). This fluorescence signature is similar to the high chlorophyll fluorescence phenotype (*hcf*) described for *Arabidopsis* mutants with defects in the photosynthetic light reactions (MILES 1982). Among the 80 insertional mutants, one strain (T78) exhibited the features of a *c*-type cytochrome deficient mutant (*ccs*). T78 accumulated low levels of holoforms of cytochrome *f* (<5%) and c_6 (<5%) similarly to a *ccsA* mutant (see Figure 2.1A) but was unaffected for the accumulation of lumen resident proteins such as plastocyanin and OEE1 or other photosynthesis proteins such as ATP synthase at the the thylakoid membrane (see Figure 2.1B). This localized the defect to cytochrome assembly and ruled out a general block in the maturation of lumenal proteins. RNA blot analyses confirmed that the expression of the *petA* and *CYC6* genes encoding apocytochrome *f* and apocytochrome c_6 respectively is unaltered in the T78 strain (data not shown). Pulse-chase experiments showed that apocytochrome *f* is synthesized in T78 but rapidly degraded, comparably to the *ccsA* mutant (see Figure 2.1C) and all of the *ccs* strains (HOWE and MERCHANT 1992; HOWE *et al.* 1995; XIE *et al.* 1998). This suggests that T78 is blocked at the step of heme attachment in the plastid lumen.

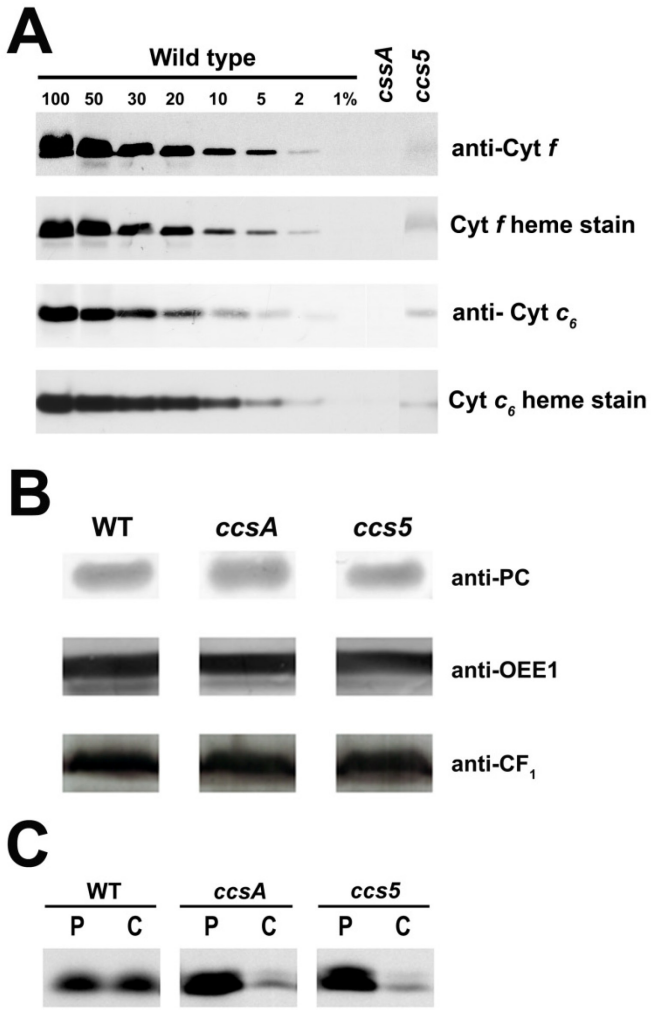


Figure 2.1. T78 displays a dual deficiency in holocytochrome *f* and c_6.

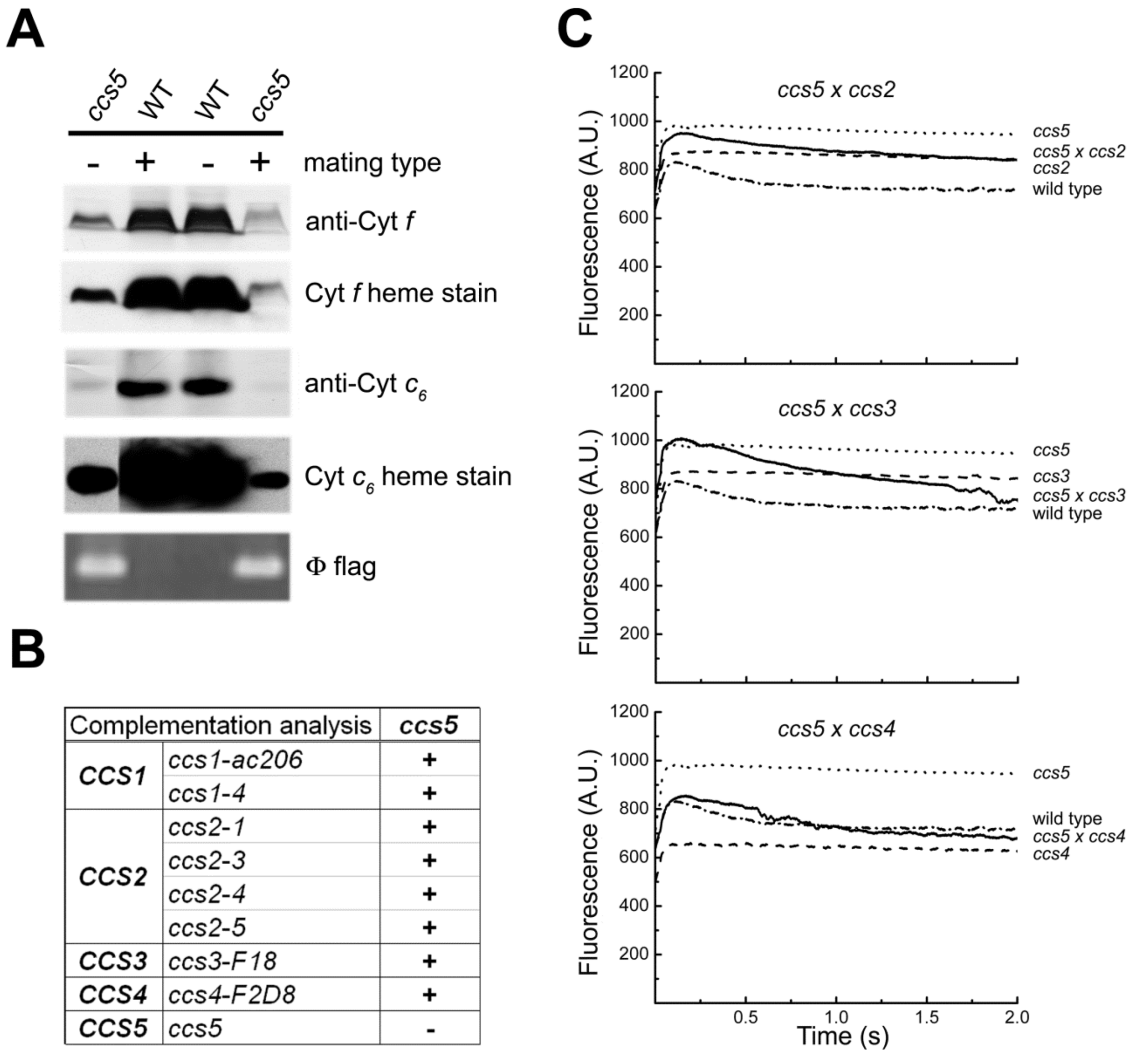
A. and B. Accumulation of plastid *c*-type cytochromes in the T78 mutant. Soluble and membrane fractions of Cu deficient cultures were analyzed for the accumulation of cytochrome *f* (anti-cyt *f* and heme stain) and cytochrome c_6 (anti-cyt c_6 and heme stain), respectively. All the samples were found to be copper-deficient based on the absence or very low abundance of immunoreactive holoplastocyanin (not shown). Samples of wild type strains (WT), T78 mutant (*ccs5*) and B6 (*ccsA*) corresponding to 5 μg of Chl were separated in SDS-containing acrylamide (12%) gels or native acrylamide gels (15%) to detect cytochrome *f* and cytochrome c_6 respectively. For an estimation of the cytochrome abundance, dilutions of the wild type sample (*arg7cw15*$_A$ for cytochrome *f* and CC125 for cytochrome c_6) were loaded on the gel. Gels were then transferred to PVDF membranes following electrophoresis before heme staining by chemiluminescence and immunodecoration with antisera against *Chlamydomonas* cytochrome *f* and cytochrome c_6 in A. and with anti-sera against plastocyanin, OEE1, ATPase CF_1 in B.

continued

Figure 2.1. continued.
C. *In vivo* synthesis and degradation of apocytochrome *f* in the *ccs5* mutant. The synthesis of cytochrome *f* was assessed by immunoprecipitation of anti-cytochrome *f* reactive polypeptides from solubilized acetone extracts of wild type (WT), B6 (*ccsA*) and T78 (*ccs5*) cells labeled for 10 min with $Na_2{}^{35}SO_4$, (P for Pulse). The labeled cells were sampled 40 min later after further incubation in the presence of unlabeled sulfate and chloramphenicol to assess the fate of the newly synthesized protein (C for Chase). Immunoprecipitates were resolved by denaturing gel electrophoresis followed by fluorography.

Tetrad analysis of T78 crossed to a wild type strain revealed that the cytochrome *f* and c_6 deficiency co-segregated as a single Mendelian trait (see Figure 2.2A). Analysis of a total of 25 mutant progeny showed a tight linkage between the cytochrome *c* deficiency and the *Φ* molecular tag in the *ARG7 Φ* insertional marker ($p>0.05$) (see Figure 2.2A). Based on the genetic analyses, we concluded that the mutation in T78 segregated as a single *CCS* locus that is tagged with the *ARG7Φ* marker. In order to determine if the *ccs* mutation in the T78 strain defines an allele of an existing or new *CCS* locus, we undertook an allelism test by performing complementation analysis. Complementation can be assessed by testing the fluorescence rise and decay kinetics of diploid zygotes (BENNOUN *et al.* 1980; GOLDSCHMIDT-CLERMONT *et al.* 1990). We reasoned that if the *ccs* mutation in T78 defines a new *CCS* locus, we should observe complementation of the CCS phenotype in zygotes resulting from the cross of strains carrying the *ccs* mutation isolated in T78 by the genetically defined *ccs* mutants. All zygotes resulting from the cross of the *ccs* mutation to strains carrying *ccs1-ac206*, *ccs1-4*, *ccs2-1*, *ccs2-3*, *ccs2-4*, *ccs2-5*, *ccs3-F18* and *ccs4-F2D8* alleles exhibited fluorescence curves with a decay phase indicative that the b_6f function was restored (see Figure 2.2BC). These results established that the affected *CCS* locus in T78 is distinct from *CCS1*, *CCS2*, *CCS3* and *CCS4*.

Moreover, based on the fact that the *CCS1* locus was found to be intact by Southern blot analysis in the T78 strain, we confirmed that this strain does not carry a molecular lesion in the *CCS1* gene (not shown). Therefore, we concluded that the T78 mutant defines yet a fifth nuclear *CCS* locus and the *ccs* allele has been named *ccs5-1::ARG7*.



B

Complementation analysis		*ccs5*
CCS1	*ccs1-ac206*	+
	ccs1-4	+
CCS2	*ccs2-1*	+
	ccs2-3	+
	ccs2-4	+
	ccs2-5	+
CCS3	*ccs3-F18*	+
CCS4	*ccs4-F2D8*	+
CCS5	*ccs5*	-

Figure 2.2. The T78 mutant defines a new *CCS* locus (*CCS5*).

A. Monogenic Mendelian segregation of the *ccs* mutation in T78. Two spores displaying the photosynthetic deficient phenotype (*ccs5*) (left: T78.5a- and right: T78.5d+) and two

continued

Figure 2.2. continued.
wild type spores (WT) from a same tetrad were used to analyze accumulation of plastid cytochromes *c* by heme stain and immunoblotting. The tetrad originates from a cross of the T78 insertional mutant to a wild type strain (CC621). The mating type mt^+ or mt^- of each spore is indicated. Segregation of the Φ flag in the progeny was followed by PCR amplification from total genomic DNA samples of the molecular tag using diagnostic primers.

B. Complementation of *ccs5* mutant with strains carrying alleles of the *CCS1*, *CCS2*, *CCS3* and *CCS4* loci. Diploid zygotes were obtained by crossing ccs5 mutant strains by strains carrying the *ccs1-ac206*, *ccs1-4*, *ccs2-1*, *ccs2-3*, *ccs2-4*, *ccs2-5*, *ccs3-F18*, *ccs4-F2D8*. "+" indicate a positive complementation based on the presence of the decay phase in fluorescence induction experiments. "–"indicates a negative complementation.

C. Fluorescence kinetics indicates restoration of cytochrome b_6f in the diploid zygotes. Fluorescence induction and decay kinetics of the *ccs5* x *ccs2-5*, *ccs5* x *ccs3-F18*, *ccs5* x *ccs4-F2D8* diploid zygotes, respective parental strains (see B.) and wild type strain (*arg7* cw_{15A}). The Fluorescence transients were measured using a home-made device. The fluorescence is in arbitrary units (A.U.) and recorded over a 2s illumination period. The rise and plateau curve for the *ccs* mutants is the signature of a specific block in electron transfer at the level of 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 electron transfer through cytochrome b_6f, an increase in the chlorophyll fluorescence is observed. Note the absence of the decay phase corresponding to reoxidation of the quinone pool (the primary electron acceptor of photosystem II) by the cytochrome b_6f complex. In the complemented diploid zygote, the presence of the decay phase indicates restoration of the b_6f complex function.

In this study, we report the identification of another *CCS* locus, *CCS5*, controlling plastid *c*-type cytochromes assembly in *Chlamydomonas reinhardtii*. The *CCS5* gene encodes the ortholog of *Arabidopsis* thioredoxin-like HCF164. The placement of CCS5 in a thiol disulfide-reducing pathway for plastid cytochrome *c* assembly is supported by the finding that a) the *ccs5* mutant is rescued by exogenous thiols, b) the soluble domain of CCS5 interacts with apoforms of cytochrome *f*, c_6 and c_{6A} in a yeast two-hybrid assay, c) a recombinant form of CCS5 is able to reduce a disulfide in apocytochrome *f* C*XX*CH

motif and d) expression of *Arabidopsis HCF164* in the chloroplast genome can complement *Chlamydomonas ccs5* mutant. We discuss the operation of thiol-disulfide chemistry on the lumenal side of the thylakoid membrane.

2.3. Experimental procedures

2.3.1. Strains and culture conditions

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/m^2/s) for *ccs* strains or under standard illumination for wild type strains (125 to 500 μmol/m^2/s) as described in (HOWE and MERCHANT 1992).

2.3.2. Insertional mutagenesis and identification of the *ccs5* mutant

The *arg7 cw15*$_A$ *mt-* strain was used as a recipient strain for insertional mutagenesis. 2 μg of pARG7.8Φ3 DNA (GUMPEL *et al.* 1994) was used to perform glass bead transformation (KINDLE 1990) and 1.6 x 10^4 arginine prototrophic transformants were selected under dim light. Insertional mutants were screened on a fluorescence video imaging system (FENTON and CROFTS 1990) at the University of Geneva. Candidate mutants displaying a rapid fluorescence induction that failed to relax, a characteristic of a block in electron transfer on the reducing side of PSII were further analyzed by immunoblotting for holocytochrome *f* accumulation.

2.3.3. Molecular genetic analysis of the T78 strain

The T78 mutant was crossed to a wild type 137 derivative strain to generate T78.3+. Tetrad analysis of T78.3+ by wild type CC621 (NO-) crosses showed that the CCS phenotype segregated as a monogenic trait along with the Φ flag present in the integrated *ARG7Φ* insertional marker (GUMPEL *et al.* 1994). The Φ flag was detected by diagnostic PCR using the phi1 (5'-GTCAGATATGGACCTTGCT-3') and phi2 (5'-CTTCTGCGTCATGGAAGCG-3') primers and genomic DNAs as templates. Spores T78.5a- and T78.5d+ come from the same tetrad in the cross of T78.3+ to wild type. T78.15b- originated from an incomplete tetrad and was retained for its ability to mate well. For the DTT-dependent rescue, T78.15b- was crossed to a *cw*- wild type strain and over 50 *ccs5* spores were tested for DTT rescue. For our analysis, we retained PH9-25 that showed the best DTT-dependent rescue of the photosynthetic growth.

Complementation analyses for test of allelism were done as described in (HARRIS 1989). Fluorescence induction kinetics were analyzed on the lawn of meiotic zygotes resulting from the cross of *ccs5* mutant strains (T78.5d$^+$, T78.5a$^-$) to strains carrying the *ccs1-ac206* (ac206.11$^-$) *ccs1-4* (CF951.15$^+$), *ccs2-1* (CF139.3$^-$), *ccs2-3* (CF521.1a$^-$), *ccs2-4* (CF779.B1$^-$), *ccs2-5* (CF854.141$^-$) *ccs3-F18* (F18.a2$^-$) and *ccs4-F2D8* (F2D8-9$^-$) alleles. The *in vivo* chlorophyll fluorescence induction kinetics of dark adapted colonies or diploid zygotes were recorded with a home-made fluorescence imaging system (Dr. D. Kramer, Washington State University, Pullman, WA) or with the FluorCam 700 MF (Photon Systems Instruments Ltd.). The colonies were illuminated with the home made system for 2 s with 30 μmol/m^{2}s of red light (640 nm) from light-emitting diodes (HLMP

C116, Hewlett Packard) and the emitted fluorescence was captured immediately by a CCD camera (Cohu, 2122–1000) in conjunction with a PIXCI-SV4 imaging board and XCIP software (EPIX Incorporated, Buffalo Grove, IL). For the FluorCam, illumination was 3 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.

2.3.4. Molecular complementation of the *ccs5* mutant strain

Electroporation was used as a transformation procedure (SHIMOGAWARA *et al.* 1998) and the T78.15b- strain was chosen as a recipient strain. For transformation experiments, an *aphVIII* PCR fragment generated with primers aph8-R (5'-CTGGGTACCCGCTTCAAATA-3') and aph8-F2 (5'-TCAGGCAGACGGGCAGGTG-3') and pSL18 (DEPÈGE *et al.* 2003) as a template was used. The *aphVIII* PCR fragment was used in a co-transformation experiment with a *CCS5* PCR fragment encompassing the entire ORF and 150 bp upstream of the ATG and 180 bp downstream of the stop codon. The *CCS5* PCR fragment was amplified from a wild type genomic DNA using primers HCF164-18 (5'- CGAAACACCAAGGTTCTGAGAGC-3') and HCF164-28 (5'-GCTTGTCAGATGTG CCCAAGC-3').

2.3.5. RNA preparation and RT-PCR experiments

RNAs were extracted as described in (QUINN and MERCHANT 1998) and reverse-transcribed into cDNAs with MMLV reverse-transcriptase or the 5'RACE kit from Invitrogen. An additional exon at the 5' end of the *CCS5* gene was identified *via* PCR

using primers HCF164-6 (5'- AGTTGGCGTAGAACTCCACCAG-3') and HCF164-24 (5'- GCAGAC GAGTGTGTCAACCTATC-3') and cDNAs as a template. The *CCS5* cDNA was synthesized with HCF164-30 (5'- GCCAATTCCCGCATCGCTTCC-3') as a *CCS5* mRNA specific primer and then used as a template in a semi-nested PCR reaction. Primers HCF164-36 (5'-GACTCCAGTGTGGCCAGCGTGG-3') and HCF164-32 (5'-GTTTCGCCAAGTTACATATTG-3') were chosen in the first PCR reaction and primers HCF164-37 (5'-CCTGGGAAGGTAGCTGCTCAGC-3') and HCF164-32 were used in the second PCR reaction. The start codon was identified by sequencing of the PCR product. A stop codon in the same reading frame was found 41 nucleotides upstream. The *CCS5* sequence has been deposited in Genbank under ADC32800.

2.3.6. Protein preparation and analysis

Supernatant and pellet fractions were obtained by freeze-thaw fractionation. Fractions were electrophoretically separated and plastid *c*-type cytochromes were revealed by immunodetection or a heme staining procedure (LEON and FERNANDEZ 2007; MILES 1982). Polyclonal antisera raised against *C. reinhardtii* cytochrome c_6, cytochrome *f* GST-fusion protein, CCS5 (this work), OEE1, CF_1 and plastocyanin were used for immunodetection by alkaline phosphatase-conjugated secondary antibodies. Pulse chase experiments to monitor the synthesis of apocytochrome *f* were performed as described previously (HOWE and MERCHANT 1992; HOWE and MERCHANT 1993; XIE *et al.* 1998).

2.3.7. Expression and purification of the soluble form of CCS5

The DNA sequence encoding the soluble domain of *Chlamydomonas* CCS5 was codon-optimized for expression in *Escherichia coli* using the DNA2.0 Gene Designer Software (VILLALOBOS *et al.* 2006). The optimized sequence was synthesized (Mr Gene, Germany) and cloned in pSK plasmid. The resulting plasmid (pMK-RQ) was used as a template in a PCR reaction with *Nde*I and *Xho*I engineered oligonucleotides as primers (HCF164 O/F Nde1: 5'- GAAGGAGATATACATATGGGTGCTCCAACTCTGGCT-3' and HCF164 O/R Xho1: 5'GTGGTGGTGGTGCTCGAGTGCGTGGTCACGTGGCA TC-3'). The PCR product was cloned into the *Nde*I/*Xho*I sites of the hexahistidinyl tag vector pET24b (Novagen) using the In-Fusion^{TM} cloning kit (Clontech), resulting in the pET24b/HCF164$_{opt}$ plasmid. For expression of the recombinant His$_6$-tagged protein, 1 liter LB broth (with 30 µg/ml kanamycin) of *Escherichia coli* strain BL21(DE3) (Novagen) carrying pET24b/HCF164$_{opt}$ was grown from a 20 ml LB broth (with 30 µg/ml kanamycin) overnight starter culture. To induce the recombinant protein, IPTG was added to a final concentration of 0.5 mM at OD_{600}=0.4 and the culture were further grown for 3 hours at 37^{o}C. Cells were then harvested by centrifugation at 4,000 g for 20 min at 4^{o}C and the pellet was stored at -20^{o}C. Batch purification of the His$_6$-tagged protein was performed under native condition using the NI-NTA resin (Qiagen). Recombinant CCS5 was used as an antigen to raise a polyclonal antibody (Covance). Immunoaffinity purification of the anti-CCS5 polyclonal was performed using Affi-Gel 10 and 15 according to the instructions of the manufacturer (Bio-Rad).

2.3.8. Disulfide reductase assay

The recombinant soluble domain of wild type CCS5 was assayed for disulfide reductase activity by using insulin properties. As insulin reduction proceeds, a precipitate is formed from the free B chain, which can be monitored at 650 nm for the change in turbidity (HOLMGREN 1979; LENNARTZ *et al.* 2001). The reaction mixture was prepared in a stirred and thermostatted (25^{o}C) cuvette with a final volume of 1 ml containing 100 mM sodium phosphate (pH 7.0), 2 mM EDTA, 1 mg/ml bovine insulin. The reaction was initiated by adding 330 μM DTT. The nonenzymatic reduction of insulin by DTT only and the reduction of insulin by 10 μM recombinant CCS5 without DTT served as negative controls. As a positive control, 2 μM thioredoxin from *Spirulina sp* (Sigma) was used.

2.3.9. Yeast two-hybrid experiments

The soluble domain of *Chlamydomonas* CCS5 (G_{206} to A_{254}, see Figure 2.4A) was used as bait and the corresponding sequence cloned *via* In-Fusion^{TM} technique (Clontech) as a PCR fragment at the *Nde*I/*Sal*I sites of the pGBKT7 vector. The sequence corresponding to the soluble domain of wild type CCS5 was PCR amplified using PHCF164BD-F (5'-GATCTCAGAGGAGGACCTGCAAGGTGCTCCAACTCTGGCTACTC-3') and PHCF164BD-R (5'-TGCGGCCGCTGCAGGTCGAGCTGCGTGGTCACGTGGCATCG-3') as primers and pET24b/$HCF164_{opt}$ as a template. Plasmids expressing the mutant forms of CCS5 (WCXXS and WSXXS) were constructed *via* Quick Change II site-directed mutagenesis kit (Stratagene). *Chlamydomonas* apocytochrome *f* without the membrane anchor (Y_{32} to R_{281}) was used as prey and the corresponding sequence cloned

via In-Fusion™ as a PCR fragment at the *Nde*I/*Xho*I of the pGADT7 vector. The sequence corresponding to apocytochrome *f* was PCR amplified using PcytfAD-F (5'-CGACGTACCAGATTACGCTCAATACCCTGTATTTGCACAAC-3') and PcytfAD-R (5'-CGATTCATCTGCAGCTCGAGCACGAGCAGGGTTTTGTAATAC-3') as primers and *Chlamydomonas* wild type genomic DNA as a template. *Chlamydomonas* apocytochrome c_6 (A_{59} to stop) was used as prey and the corresponding sequence cloned *via* In-Fusion™ as a PCR fragment at the *Nde*I/*Xho*I of the pGADT7 vector. The sequence corresponding to apocytochrome c_6 was PCR amplified using $Pcytc_{6opt}$AD-F (5'-CGACGTACCAGATTACGCTCAAGCGGATCTGGCCCTGGGCGCACAGG-3') and $Pcytc_{6opt}$AD-R: (5'-CGATTCATCTGCAGCTCGAGCGTATTTCCACGCAGCAT CGGTAGC-3') as primers and the pMA/cytc_6 plasmid carrying the codon-optimized cytochrome c_6 encoding DNA as a template. *Chlamydomonas* apocytochrome c_{6A} (A_{46} to stop) was used as prey and the corresponding sequence cloned *via* In-Fusion™ as a PCR fragment at the *Nde*I/*Xho*I of the pGADT7 vector. The sequence corresponding to apocytochrome c_{6A} was PCR amplified using $Pcytc_{6Aopt}$AD-F (5'-CGACGTACCAGAT TACGCTCAAG CTGTTGCAATTGCAGCTGCTGCTGC-3') and $Pcytc_{6Aopt}$AD-R: (5'-CGATTCATCTGCAGCTCGAGCAGATTTCCAACCAGCAGCAGCACG-3') as primers and the pMA/cytc_{6A} plasmid carrying the codon-optimized cytochrome c_{6A} encoding DNA as a template. The codon-optimized version of cytochrome c_{6A} has been determined using the DNA2.0 Gene Designer Software (VILLALOBOS *et al.* 2006) and synthesized by Mr Gene. The yeast strain PJ69-4A was used as a reporter (JAMES *et al.* 1996) and β-galactosidase activity was measured as described in (MEYER *et al.* 2005).

2.3.10. *In vitro* redox assay

The DNA sequence encoding the soluble domain of *Chlamydomonas* apocytochrome *f* without the membrane anchor was codon-optimized for expression in *E. coli* using the DNA2.0 Gene Designer Software (VILLALOBOS *et al.* 2006). The optimized sequence was synthesized (Mr Gene, Germany). The resulting plasmid (pMK-CYTF) was used as a template in a PCR reaction with *Nde*I and *Xho*I engineered oligonucleotides as primers (Solcytfopt-F: 5'-CTTTAAGAAGGAGATATACATATGTACCCGGTTTTCGCTCAG CAGAACTA-3' and Solcytfopt-R: 5'-CGGATCTCAGTGGTGGTGGTGGTGGTGAC GTGCCGGGTTCTGCAGGACGA-3'). The PCR product was cloned into the *Nde*I/*Xho*I sites of the hexahistidinyl tag vector pET24b (Novagen) using the In-Fusion™ cloning kit (Clontech), resulting in the pET24b/$CYTF_{sol}$ plasmid. Purification of apocytochrome *f* was as described for soluble CCS5 except that IPTG concentration is 0.4 mM and that induced cells were harvested at OD_{600}=0.6. Soluble CCS5 in 25 mM Tris-HCl (pH 7.5) was reduced by 200 µM DTT during 1 hour on ice. DTT was eliminated by buffer exchange using the Amicon centriprep system (Ultracel-10 Membrane, Millipore). Air oxidized apocytochrome *f* (8µM) in 25 mM Tris-HCl (pH 7.5) was incubated for 60 min at 25 °C in the presence or absence of reduced soluble CCS5 (16µM). After incubation, proteins were precipitated with trichloroacetic acid (final 5%) and washed with ice-cold acetone, then dissolved in buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 10 mM AMS (4-acetamido-4'-maleimidylstilbene-2, 2'-disulfonic acid). After 1h30 of incubation, reduced (AMS derivative) and oxidized forms of cytochrome *f* and CCS5 were separated by 15% non-reducing SDS-PAGE and visualized by Coomassie staining.

2.3.11. Transformation of the chloroplast genome

The *Arabidopsis HCF164* ORF was codon-optimized according to the codon bias for *Chlamydomonas* chloroplast genes using the DNA2.0 Gene Designer Software (VILLALOBOS *et al.* 2006). The corresponding codon-optimized sequence (*HCF164$_{opt}$*) was synthesized by Mr Gene (Germany). The cloned *HCF164$_{opt}$* was used as a template in a PCR with the primers HCF164$_{opt}$/NdeI-F (5'-TTTAAAAAAATTAACATATG GCTCGTTTAGTATTTTC-3') and HCF164$_{opt}$/PstI-R (5'-AGCTTGCATGCCTGC AGTTAACCATGTGATAATGGATC-3'). The non-optimized sequence of the *HCF164* ORF (*HCF164*) was amplified using reverse-transcribed RNAs (extracted from *Arabidopsis* wild-type leaves) as template and HCF164/NdeI-F (5'-TTTAAAAAAATTAACATATGGCTCGCTTAGTATTTTC-3') and HCF164/PstI-R (5'-AGCTTGCATGCCTGCAGTTATCCATGGCTTAAGGGATC-3') as primers. The PCR products were cloned at the *Nde*I/*Pst*I sites of pSK.Km^R (BATEMAN and PURTON 2000) using In-Fusion^{TM} (Clontech) and the resulting plasmids are pSK-HCF164 and pSK-HF164$_{opt}$. A *Sma*I/*Eco*RI fragment containing either the *HCF164* or *HCF164$_{opt}$* ORF was cloned into the *Eco*RV/*Pst*I digested pmp-aadA plasmid (HAMEL *et al.* 2003). The resulting plasmids pmp-HCF164 and pmp-HCF164$_{opt}$ contain the *HCF164* ORF between the *atpA* promoter and *rbcL* terminator for expression in the chloroplast. The pmp plasmids contain a 5.5 kb fragment of chloroplast DNA including the *ccsA* gene. The *atpA-HCF164-rbcL* cassette is located at a neutral site upstream the *ccsA* gene (HAMEL *et al.* 2003). The *ccs5* mutant strain was co-transformed by biolistic with pmp-HCF164 or pmp-HCF164$_{opt}$ in combination with p699-aadA. The p699-aadA plasmid contains the

spectinomycin resistance cassette (*aadA*) at a neutral site between the *trnG2* and *psaB* genes and is used for selection of the transformants on the basis of spectinomycin resistance (a gift from J. Winburn and R. Tabita, Ohio State University, Columbus, OH). Co-integrants are expected to carry the *aadA* cassette between the *trnG2* and *psaB* genes and the *atpA-HCF164-rbcS* cassette upstream the *ccsA* gene. Integration of the cassettes results from homologous recombination between the chloroplast genome and the transforming plasmids introduced by biolistic. *C. reinhardtii* vegetative cells grown in TAP liquid culture (1-2 X 10^6cells/ml) were transformed by particule bombardment (using a home-made particule gun at The Ohio State University, Columbus, OH) as described in (XIE and MERCHANT 1996). Particules were coated with DNA as in (SAWANT *et al.* 2000). For the complementation experiment, T78.15b$^-$ vegetative cells were collected by centrifugation, 5 X 10^6 to 10^7 cells were plated on solid fresh TAP medium containing 150 µg/ml spectinomycin and immediately bombarded. Bombarded cells were incubated under dim light (25 µmol/m^2/s) until colonies appeared. The primary spectinomycin-resistant (Spec^R) transformants were screened for the co-integration of the *atpA-HCF164-rbcS* cassette by diagnostic PCR. Among the primary spectinomycin-resistant (Spec^R) transformants, 30% were found to also contain the *atpA-HCF164-rbcS* cassette. Three consecutive transfers of the transformants on agar plates with 700 µg/ml spectinomycin were performed to ensure homoplasmy (HAMEL *et al.* 2003).

2.4. Results

2.4.1. The *CCS5* gene product functions in redox chemistry

In bacteria, loss of cytochrome *c* assembly redox factors with a proposed reducing activity can be by-passed by provision of exogenous reduced thiols in the medium (BARDISCHEWSKY and FRIEDRICH 2001; BECKETT *et al.* 2000; DESHMUKH *et al.* 2000; ERLENDSSON and HEDERSTEDT 2002; FEISSNER *et al.* 2005). Therefore, as a preliminary functional assessment of the products of the genetically defined *CCS* loci, we tested the ability of exogenous thiols (MESNA, DTT, cysteine) to rescue the phototrophic growth of all *ccs* strains. Among the different compounds tested, we found that exogenous thiols such as DTT and MESNA could rescue the photosynthetic deficiency of the *ccs5* mutant (see Figure 2.3A and not shown). Photosynthetic competence in the chemically rescued colonies correlates with accumulation of holocytochrome *f*, which is strictly required for photosynthesis (see Figure 2.3B). Note that cytochrome c_6 is only required for photosynthesis in copper deficient conditions where it acts as a substitute for plastocyanin, a copper containing protein. For experimental convenience, we usually monitor photosynthetic growth in copper replete conditions where cytochrome *f* is the only *c*-type cytochrome required for photosynthesis. As expected, fluorescence rise and decay kinetics confirmed that DTT restores the function of cytochrome b_6f (not shown). The DTT dependent rescue of the *ccs5* mutant suggests that the gene product acts as a reductant in the cytochrome *c* assembly process.

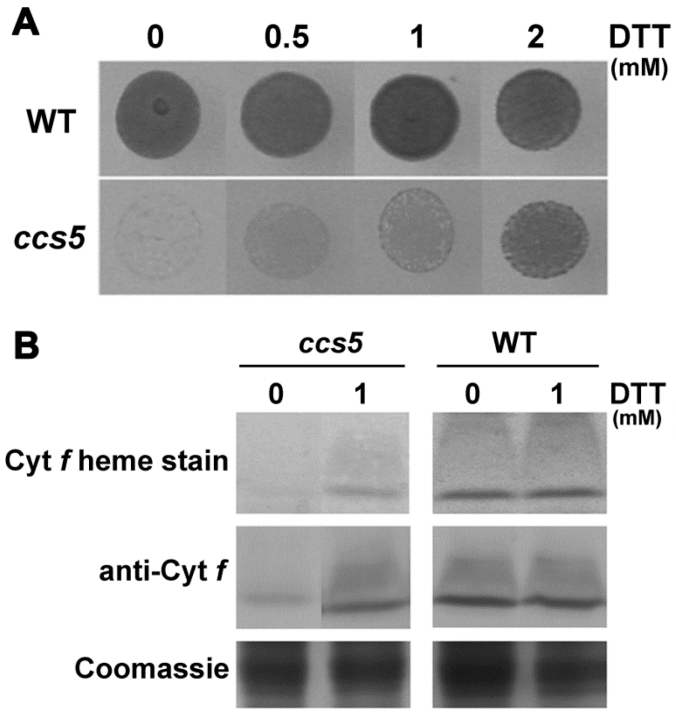


Figure 2.3. The *ccs5* mutant is rescued by exogenous thiols.

A. DTT dependent photosynthetic rescue of the *ccs5* mutant. Equal numbers of wild-type (CC124) and *ccs5* mutant (PH9-25) cells were spotted on minimal medium supplemented with DTT as indicated, and incubated at 25 °C at a light intensity of 500 μmol/m^2/s for 12 days.

B. DTT dependent restoration of holocytochrome *f* assembly in the *ccs5* mutant. In gel cytochrome *f* heme staining (QUINN and MERCHANT 1998) and anti-cytochrome *f* immunoblot analyses were performed on total protein extracts from the *ccs5* mutant (PH9-25) and wild type (WT) strains. Cells were grown heterotrophically (acetate, low light) in absence or presence of 1 mM DTT. Coomassie blue staining is used to show equal loading. Note that cells grown heterotrophically showed the best rescue with 1 mM DTT.

2.4.2. *CCS5* encodes a thioredoxin-like protein

In order to identify the *CCS5* gene, we searched the *Chlamydomonas* genome for candidate proteins based on the occurrence of 1) a redox active domain (*e.g.*C*XX*C) and 2) an algorithm-predicted targeting sequence to the plastid. Our approach was greatly

facilitated by the detailed annotation of the *Chlamydomonas* genome (MERCHANT *et al.* 2007). Several candidate proteins were retrieved and we focused our attention on a thioredoxin-like protein because of its sequence similarity to HCF164, a lumen-located membrane anchored thioredoxin-like protein involved in cytochrome b_6f complex biogenesis in *Arabidopsis* (see section 1.4.2.3) (LENNARTZ *et al.* 2001; MOTOHASHI and HISABORI 2006). Because the *ccs5* mutant is an insertional mutant, we reasoned that the physical integrity of the gene that encodes the HCF164-like protein should be altered in the mutant strain if *ccs5* is deficient for this protein. PCR-based scanning of the locus confirmed that up to 3 kb of the corresponding locus, including the entire coding sequence is deleted in the *ccs5* strain (not shown). This alteration in the *ccs5* mutant presumably occurred upon insertion of the *ARG7* insertional marker, a common occurrence in *Chlamydomonas* (LEON and FERNANDEZ 2007). In addition, qPCR experiments showed that the transcript is detected in wild type but not in the *ccs5* strain (not shown). We concluded that *ccs5* is a null-mutant of the gene encoding the HCF164-like protein. Because only a partial EST sequence was available in the database, we sought to confirm the 5' end of the *CCS5* gene model and determine the initiation codon by performing RT-PCR experiments. A full length cDNA was assembled and shown to encode a 254 amino acid protein with a predicted plastid targeting sequence (see Figure 2.4A). The CCS5 protein is 35% identical to *Arabidopsis* HCF164. From similarity to HCF164, CCS5 is predicted to be anchored to the membrane by a single hydrophobic stretch with the redox active motif exposed to the thylakoid lumen (see Figure 2.4A). Orthologs of CCS5/HCF164 are found in the green lineage and they all display a similar

topology with a N-terminal membrane anchor and the redox active domain in the C-terminal part of the protein (see Figure 2.4B).

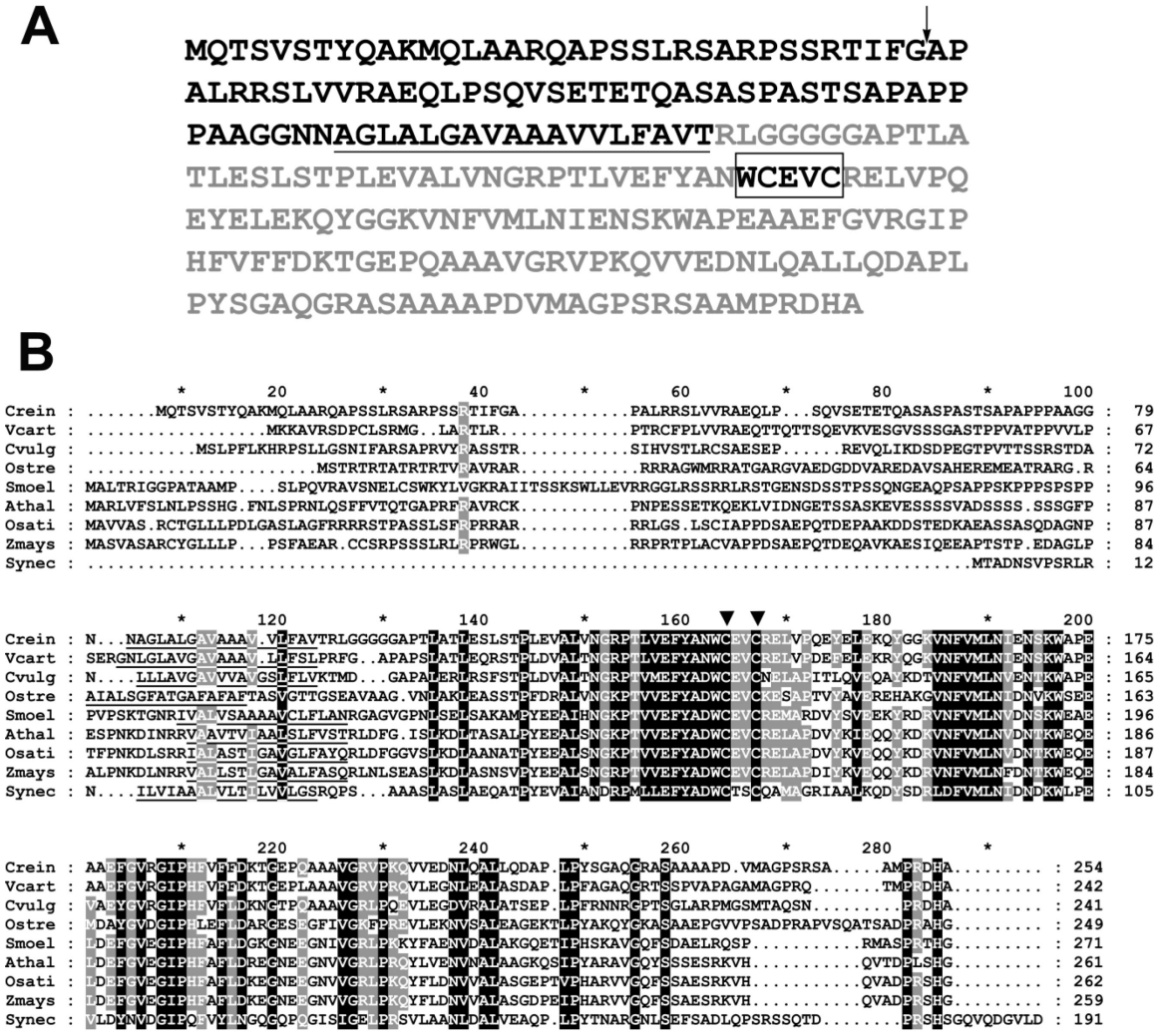


Figure 2.4. CCS5 is a thioredoxin-like protein conserved in the green lineage.

A. Amino-acid sequence of *Chlamydomonas* CCS5. The amino acid sequence of *Chlamydomonas* CCS5 protein was deduced from assembly of a full-length cDNA. The cleavage site of the plastid targeting sequence predicted by PredSL (PETSALAKI *et al.* 2006) is indicated by a black arrow and the putative transmembrane domain is underlined. The hydrophilic C-terminal domain predicted to be facing the luminal side of the thylakoid membrane is highlighted in light grey. The thioredoxin motif with active cysteines is boxed.

continued

Figure 2.4. continued.
B. Alignment of CCS5/HCF164-like proteins. Sequences of CCS5/HCF164 from *Chlamydomonas reinhardtii* (Crein), *Volvox carteri* (Vcart, 80757), *Chlorella vulgaris* (Cvulg, 79381), *Ostreococcus* RCC809 (Ost, 68525), *Selaginella moellendorffii* (Smoel, 159329), *Arabidopsis thaliana* (Athal, CAC19858), *Oriza sativa* (Osati, NP_001051387), *Zea mays* (Zmays, NP_001152226) and TxlA from *Synechococcus sp*. PCC 7942 (Synec, U05044) were aligned using the CLUSTALW algorithm (Blosum62 scoring matrix) in Bioedit. The alignment was edited using the GeneDoc multiple alignment editor. Amino-acids strictly conserved in all sequences are shaded in black and those conserved in the majority of the sequences (7 of 9) are shaded grey. The putative membrane anchor is underlined. Two downward arrows indicate the cysteines in the WC*XX*C motif.

To confirm that the *ccs5* mutation affects the *CCS5* gene, we performed complementation experiments. The *ccs5* mutant strain was co-transformed with two PCR products corresponding to the *aphVIII* selectable marker conferring paromomycin-resistance (Pm^R) and a 2.5 kb fragment containing the entire *CCS5* gene. Pm^R transformants were selected and screened for restoration of photosynthesis on the basis of fluorescence. Out of the 211 Pm^R transformants that we screened, 23 showed wild type like fluorescence, suggesting that b_6f and hence cytochrome *f* assembly was restored (see Figure 2.5A). Consistently, the transformants displaying a wild type like fluorescence rise and decay kinetics were able to grow photoautotrophically and accumulate wild type level of holocytochrome *f* and c_6 (see Figure 2.5BC). We also showed that CCS5 protein accumulation was restored in the complemented transformants (see Figure 2.5C). However the two independent transformants that we analyzed did not show wild type level of CCS5 protein despite the fact that cytochrome *f* and c_6 accumulated to wild type level. The different level of accumulation of CCS5 might reflect the differential expression of the transforming DNA at the integration site in the chromosome. This suggests that CCS5 is probably not limiting for the assembly process. As expected, all of

the 68 Pm^R transformants that were only transformed with the *aphVIII* PCR product exhibited b_6f-minus fluorescence rise and decay kinetics (see Figure 2.5A).

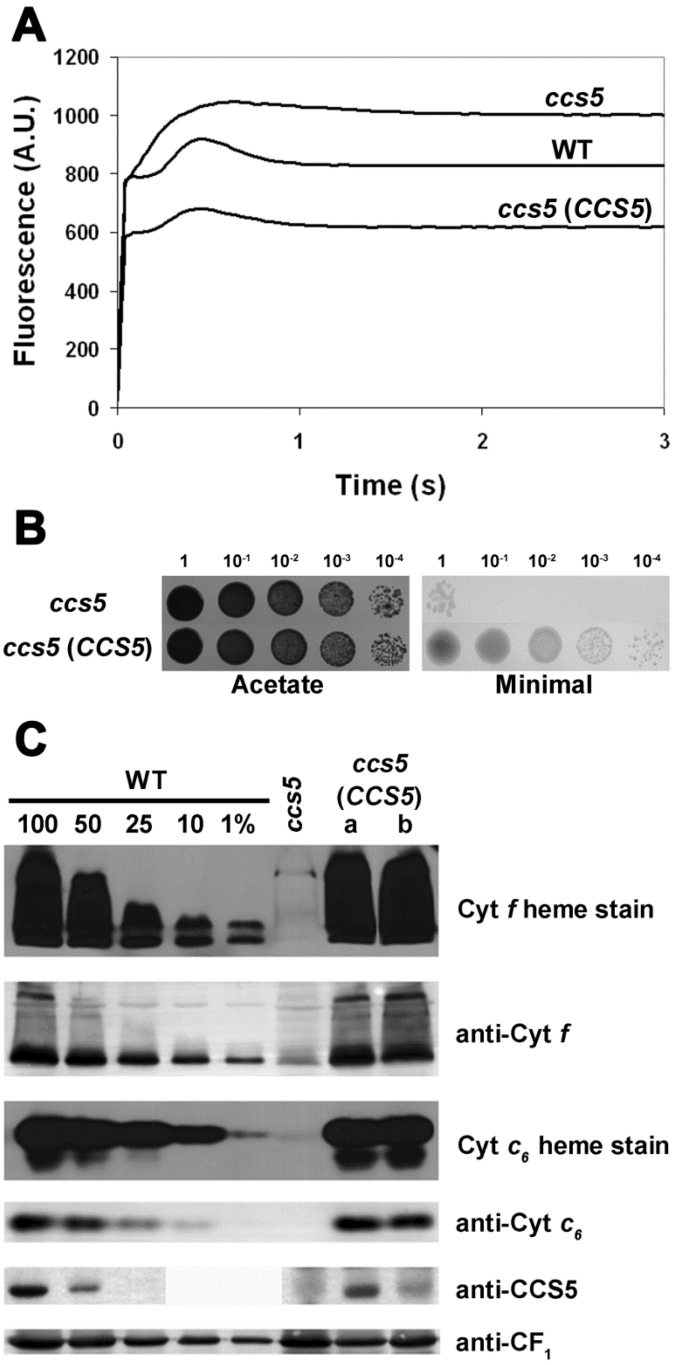


Figure 2.5. The thioredoxin-like encoding gene complements the *ccs5* mutant.

continued

Figure 2.5. continued.
For ABC, the *ccs5* mutant (T78.15b-) carries the Pm^R-cassette and the *CCS5* complemented transformants result from the co-transformation of the Pm^R cassette and the *CCS5* gene.

A. Fluorescence kinetics indicate restoration of cytochrome b_6f in the complemented *ccs5* mutant. Fluorescence induction and decay kinetics of the *ccs5*, wild type, and *ccs5* complemented with the wild type *CCS5* gene. One representative transformant (#a) is shown. Fluorescence transients were measured on colonies grown for 2 days on solid TAP medium after a short dark adaptation using Fluorcam 700 from Photon Systems Instruments. The fluorescence is in arbitrary units (A.U.) and recorded over a 3s illumination period.

B. The *CCS5* gene restores phototrophic growth to the *ccs5* mutant. Ten fold dilution series of *ccs5* and a representative *ccs5* transformant (#a) carrying the *CCS5* gene have been plated on acetate (heterotrophic conditions, 20 µmol/m^2/s of light) and minimal medium (phototrophic conditions, 500 µmol/m^2/s of light) and incubated for 1 week at 25°C.

C. Plastid *c*-type cytochromes accumulation is restored in the *CCS5* complemented *ccs5* strain. Wild type (CC124), *ccs5* and two independent *ccs5* transformants (#a and #b) carrying the *CCS5* gene were analyzed for cytochrome *f* and cytochrome c_6 accumulation via heme stain and immunoblot analyses. Samples corresponding to 18 µg of chlorophyll were separated in 12% SDS acrylamide gel to detect cytochrome *f* , CCS5 and CF_1 of the ATPase that serves as a 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 *ccs5* complemented strain, dilutions of the wild type sample were loaded on the gel. Gels were transferred to PVDF membranes before heme staining by chemiluminescence and immunodetection with antisera against *Chlamydomonas* cytochrome *f*, cytochrome c_6, CCS5 or CF_1.

2.4.3. CCS5 displays disulfide reductase activity

The DTT dependent rescue of the *ccs5* mutant and the fact that CCS5 displays a thioredoxin-like motif speaks to a reducing activity for this protein in the assembly process of holocytochrome *c*. One hypothesis is that CCS5 is required to reduce the cysteines in the C*XX*CH motif prior to the heme attachment reaction. In such a working

model, CCS5 would act as a disulfide reductase. To demonstrate such an activity, we purified the soluble domain of CCS5 (see Figure 2.4A) as a recombinant protein and tested its disulfide reductase activity in the insulin reduction assay (HOLMGREN 1979; LENNARTZ *et al.* 2001; MOTOHASHI and HISABORI 2006). The insulin reduction assay is a quantitative assay that measures the rate of insulin reduction spectrophotometrically at 650 nm as turbidity formation from the precipitation of the free insulin B chain release after reduction of a structural disulfide bond. As shown on Figure 2.6, CCS5 is active in disulfide reduction, similarly to the stromal thioredoxin from the alga *Spirulina sp.* that is known to display disulfide reductase activity (HOLMGREN 1979). We concluded that CCS5 exhibits disulfide reductase activity.

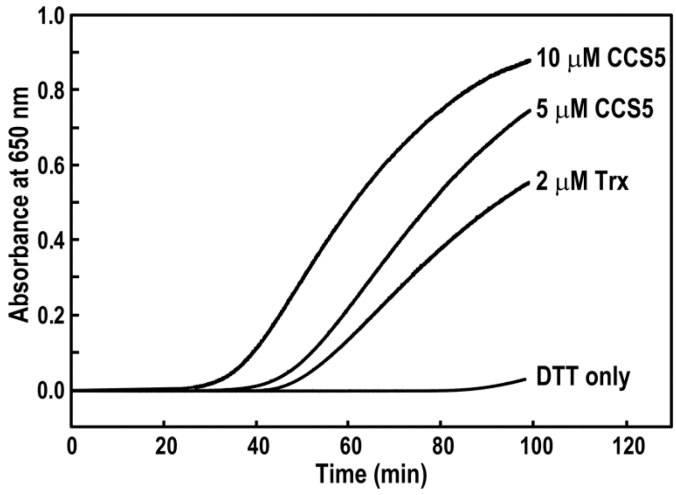


Figure 2.6. Recombinant soluble CCS5 displays disulfide reductase activity.

The disulfide reductase activity of recombinant CCS5 was measured by using the insulin reduction assay with 5 μM and 10 μM protein as described under "Experimental Procedures". As a positive control, 2 μM thioredoxin (Trx) from *Spirulina sp* were used. The nonenzymatic assay with DTT only served as a negative control. No reduction of insulin was observed with 10 μM recombinant CCS5 in absence of DTT (data not shown).

2.4.4. CCS5 interacts with apocytochrome *f* and c_6

To provide evidence that plastid apoforms of cytochrome *c* are the relevant targets of action of CCS5 *in vivo*, we aimed to demonstrate interaction between the thioredoxin-like domain of CCS5 and the domain of apocytochrome *f* and c_6 carrying the C*XX*CH heme binding site. We first chose to evidence such an interaction using apocytochrome *f* as a prey and CCS5 as bait in a GAL4-based two-hybrid system. As shown in Figure 2.7, CCS5 and apocytochrome *f* are able to interact based on the recovery of GAL4-dependent adenine/histidine prototrophies and β−galactosidase activities in the yeast reporter strain. Next, we address the importance of the cysteine residues in CCS5 for such an interaction. In thioredoxin proteins, the first cysteine residue of the WC*XX*C motif attacks the disulfide in a target and forms a mixed disulfide that is resolved by the second cysteine of the motif (MEYER *et al.* 2009). Mutation of the resolving cysteine or both cysteines in the WC*XX*CH did not abolish interaction between the thioredoxin-like domain and apocytochrome *f* (see Figure 2.7). Compatible with its function in *c*-type cytochrome assembly, CCS5 also interacts with apocytochrome c_6 in the yeast two-hybrid assay (see Figure 2.7). Similarly to our finding with apocytochrome *f*, the cysteines of the thioredoxin motif in CCS5 are not required for such an interaction (not shown).

We concluded that the cysteines of the thioredoxin motif are not absolutely required for CCS5 to interact with apocytochrome *f* and c_6, its relevant targets of action in *vivo*.

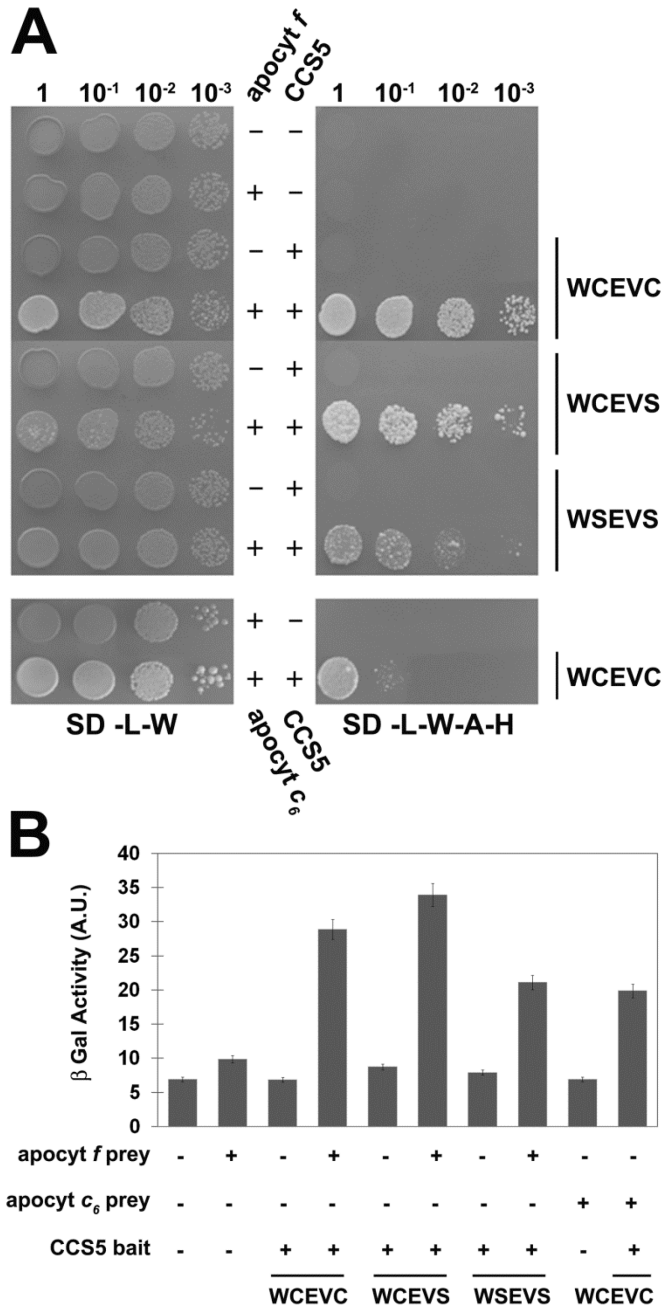


Figure 2.7. CCS5 interacts with apocytochrome *f* and c_6 in a yeast two-hybrid assay.

The soluble domain of *Chlamydomonas* wild type (WCEVC) and cysteine-less CCS5 (WCEVS and WSEVS) constitutes the bait and is expressed as a fusion with the GAL4 DNA binding domain (BD) from the *TRP1*-based pGBKT7 vector. The soluble domain of *Chlamydomonas* apocytochrome *f* or c_6 constitutes the prey and is expressed as a fusion with the GAL4 activation domain (AD) from the *LEU2*-based pGADT7 vector.

continued

Figure 2.7. continued.
The yeast PJ69-4A reporter strain was co-transformed with various combinations of two-hybrid plasmids. "+"refers to the presence of the bait or prey expressing constructs and "–" refers to the presence of pGBKT7 or pGADT7 vectors in the yeast transformants.

A.GAL4-dependent adenine and histidine prototrophies indicate interaction between apocytochrome *f*/c_6 and CCS5. The yeast transformants were tested for adenine/histidine prototrophies that depend upon reconstitution of an active GAL4. Ten-fold dilution series of one representative transformant for each combination were plated on solid medium lacking leucine and tryptophan (SD -L-W) and lacking leucine, tryptophan, adenine and histidine (SD -L-W-A-H) and incubated at 28°C for 2 or 4 days respectively.

B. GAL4-dependent β-galactosidase activities indicate interaction between apocytochrome *f* /c_6 and CCS5. The yeast PJ69-4A transformants were tested for β-galactosidase activity that depends upon reconstitution of an active GAL4. 2-nitrophenyl β-D-galactopyranoside (o-NPG) was used as a substrate. β-galactosidase activity is expressed in arbitrary units (A.U.). The values displayed are the average β-galactosidase activities from three individual transformants with standard deviation indicated by error bars.

2.4.5. CCS5 interacts with the newly identified apocytochrome c_{6A}

The recently identified cytochrome c_{6A} in *Arabidopsis*, a new *c*-type cytochrome of the thylakoid lumen does not seem to function in the known reactions of photosynthesis contrary to cytochrome c_6 (MERCHANT and DREYFUSS 1998). Analysis of the *Chlamydomonas* genome reveals the presence of a cytochrome c_{6A} ortholog that cannot be detected by heme staining (data not shown), probably due to its low abundance. Plastid cytochrome c_{6A} is a *c*-type cytochrome, therefore like cytochrome *f* and cytochrome c_{6A}, it is expected that it is a relevant target of action of CCS5. To investigate this hypothesis, we aimed to demonstrate interaction between the thioredoxin-like domain of CCS5 and the apocytochrome c_{6A} domain carrying the C*XX*CH heme binding. As described in the above section 2.4.4, we chose to evidence such an interaction by using apocytochrome c_{6A} as a prey and CCS5 as a bait in a GAL4-based two-hybrid

system. As shown in Figure 2.8, CCS5 and apocytochrome c_{6A} are able to interact based on the recovery of GAL4-dependent adenine/histidine prototrophies and β-galactosidase activities in a yeast reporter strain. The interactions between CCS5 and apocytochrome *f* or apocytochrome c_6 have been repeated to serve as positive control. We concluded that apocytochrome c_{6A} constitues a new target of the thioredoxine-like CCS5.

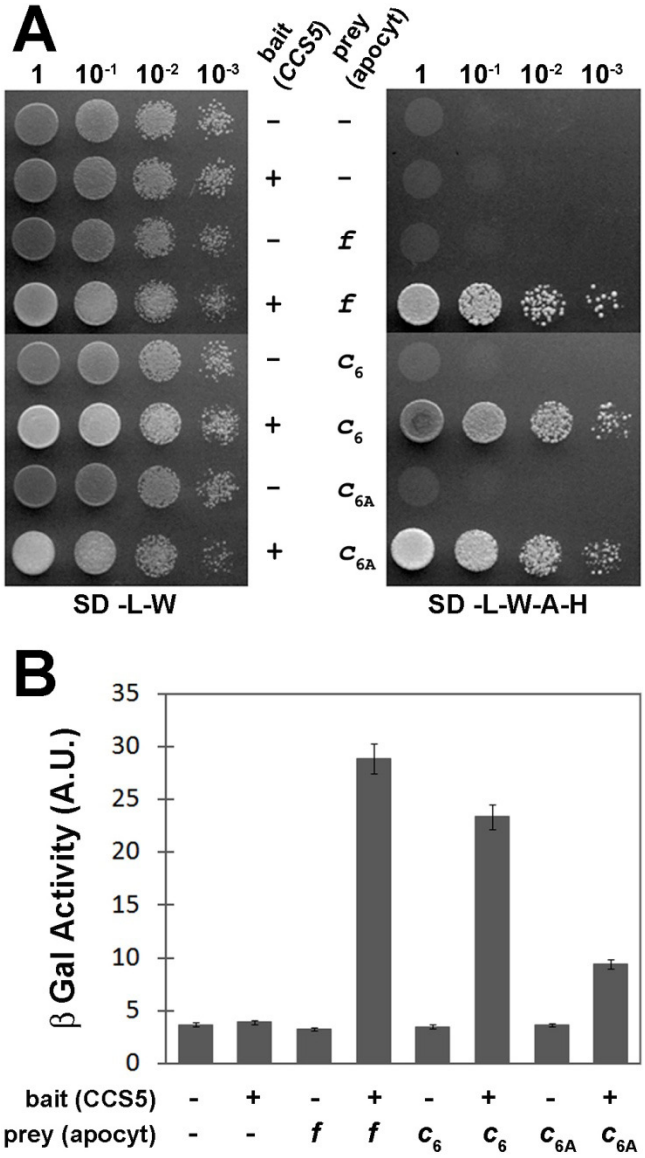


Figure 2.8. CCS5 interacts with apocytochrome c_{6A} in a yeast two-hybrid assay.

continued

Figure 2.8. continued.
The soluble domain of *Chlamydomonas* wild-type CCS5 constitutes the bait and is expressed in fusion with the GAL4 DNA binding domain from the *TRP1*-based pGBKT7 vector. The soluble domain of *Chlamydomonas* apocytochrome (apocyt) *f*, c_6 and c_{6A} constitutes the prey and is expressed as a fusion with *LEU2*-based pGAD17 vector.
The yeast PJ69-4A reporter strain was co-transformed with various combinations of two-hybrid plasmids. "+"refers to the presence of the bait or prey expressing constructs and "–" refers to the presence of pGBKT7 or pGADT7 vectors in the yeast transformants.

A. GAL4-dependent adenine and histidine prototrophies indicate interaction between apocytytochromes *f*, c_6 and c_{6A} and CCS5.

B. GAL4-dependent *β*-galactosidase activities indicate interaction between apocyto-chromes *f*, c_6 and c_{6A} and CCS5. The yeast two-hybrid experimental procedure and conditions are the same as described in Figure 2.7.

2.4.6. *In vitro* reduction of apocytochrome *f* C*XX*CH by CCS5

The fact that CCS5 exhibits disulfide reductase activity and interacts with apocytochrome *f* prompted us to develop an *in vitro* assay to test if apoforms of plastid cytochromes *c* can be acted upon by CCS5. Despite several attempts, we were unable to express apocytochrome c_6 as a recombinant protein. Hence this substrate could not be tested in our *in vitro* assay. However, we were able to express and purify a recombinant form of apocytochrome *f* that does not contain the membrane anchor and carries no other cysteines that the ones present in the C*XX*CH motif. We first tested if the disulfide bonded C*XX*CH motif in apocytochrome *f* could be chemically reduced by action of a reductant. As shown in Figure 2.9A, oxidized apocytochrome *f* can be converted to its reduced form in a dose dependent manner by the action of DTT. Next, we tested if reduced CCS5 can catalyze the reduction of oxidized apocytochrome *f*. DTT treatment of CCS5 resulted in full reduction of the protein (not shown). However, upon DTT removal,

we noticed that a fraction of reduced CCS5 is converted back to its oxidized form (see Figure 2.9B, lane 4). Incubation of CCS5 with the apocytochrome substrate resulted in the conversion of oxidized to reduced apocytochrome *f* (see Figure 2.9B lane 2). As expected if oxidized apocytochrome *f* is reduced via CCS5-mediated thiol-disulfide exchange, we noted a conversion of reduced CCS5 to its oxidized form (see Figure 2.9B, lane 3).

Quantification of the oxidized and reduced species indicates that one molecule of oxidized apocytochrome *f* reacted with one molecule of reduced CCS5 enzyme. Extended incubation time (from 1h to 3h) or a change in pH (4 instead of 7.5) did not increase the fraction of oxidized apocytochrome *f* that is converted to reduced by the activity of CCS5 (not shown). We concluded that the disulfide between the cysteines of apocytochrome *f* heme binding is reduced by the activity of CCS5. Hence CCS5 acts as a C*XX*CH disulfide reductase.

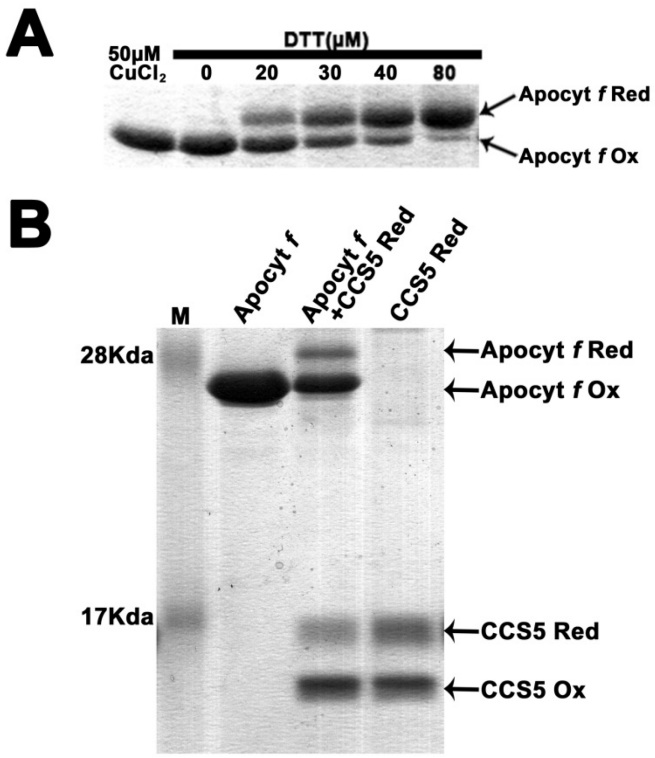


Figure 2.9. Reduction of apocytochrome *f* C*XX*C disulfide by recombinant CCS5.

AMS treated samples were separated by nonreducing SDS-PAGE (15%) and visualized by Coomassie Brilliant Blue R-250 staining. The position of reduced (Apocyt *f* Red) and oxidized (Apocyt *f* Ox) forms of apocytochrome *f*, reduced (CCS5 Red) and oxidized (CCS5 Ox) forms of CCS5 are indicated by arrows. AMS is an alkylating molecule and treatment of exposed thiols in apocytochrome *f* and CCS5 with AMS results in an increase in molecular mass of the alkylated molecules that can be detected by SDS-PAGE. Only reduced apocytochrome *f* or CCS5 reacts with AMS.

A. DTT-dependent reduction of oxidized apocytochrome *f* C*XX*CH. Recombinant apocytochrome *f* was oxidized by incubation with 50 μM $CuCl_2$. Air oxidized apocytochrome *f* was reduced by incubation with increasing concentrations of DTT during 1h.

B. CCS5-dependent reduction of oxidized apocytochrome *f* C*XX*CH. Prestained protein ladder (Fermentas) was used in lane 1 (M). Lane 2 (Apocyt *f*): Oxidized apocytochrome *f*. Lane 3 (Apocyt *f* + CCS5): Oxidized apocytochrome *f* (10 μM) was treated with recombinant reduced CCS5 (16 μM) as described under "Experimental Procedures". Lane 4 (CCS5): DTT-reduced CCS5 after DTT removal. Quantification using the Image

continued

Figure 2.9. continued.
J software indicates that 22% of oxidized apocytochrome *f* (2.2 μM) becomes reduced while 14% of reduced CCS5 (2.24 μM) is converted to its oxidized form.

2.4.7. Expression of *Arabidopsis HCF164* in the chloroplast genome of *ccs5* mutant can rescue holocytochrome *f* assembly.

Chlamydomonas CCS5 and *Arabidopsis* HCF164 proteins share 35% identity. From the known localization and topology of HCF164, it is likely that CCS5 is bound to the thylakoid membrane by a single hydrophobic stretch with the redox active motif exposed to the thylakoid lumen (LENNARTZ *et al.* 2001; MOTOHASHI and HISABORI 2006). In order to investigate if HCF164 is the functional homolog of CCS5, we chose a heterologous functional complementation approach and expressed the full length *Arabidopsis HCF164* cDNA in the chloroplast genome of the *Chlamydomonas ccs5* mutant. We reasoned that if HCF164 carries the same function as CCS5, expression of HCF164 from the plastid chromosome of the *ccs5* mutant should restore holocytochrome *f* accumulation assuming that HCF164 is properly targeted and inserted in the thylakoid membrane. A proof-of-concept for this approach had already been established by expressing the *Arabidopsis ARG9* cDNA in the chloroplast genome of *Chlamydomonas* the *arg9-2* strain (REMACLE *et al.* 2009). ARG9 is a stromal enzyme involved in arginine biosynthesis and expression of *Arabidopsis ARG9* from the plastid genome restores arginine prototrophy to the *arg9-2* mutant, demonstrating functional complementation. To by-pass the possible problem of expression of the HCF164 protein due to codon biais between *Arabidopsis* nuclear genome and *Chlamydomonas* plastid genome, a codon optimized version of the *HCF164* gene ($HCF164_{opt}$) for expression in the plastid genome has also been used for the

heterologous functional complementation experiment. Plastid transformants expressing *HCF164* or $HCF164_{opt}$ at a neutral site of the plastid genome of the *ccs5* mutant were generated as described in Material and Methods. One representative transformant was chosen for further analysis. *HCF164* or $HCF164_{opt}$ expressing transformants display wild type fluorescence and decay kinetics, suggesting that the b_6f complex and hence the cytochrome *f* assembly was restored (see Figure 2.10A). Consistently, such transformants are restored for holocytochrome *f* accumulation (see Figure 2.10B). Note that holocytochrome *f* accumulation in the *ccs5* nuclear transformants complemented with wild type *CCS5* gene is similar to that in *ccs5* plastid transformants expressing *HCF164* or $HCF164_{opt}$ (see Figure 2.10B). To monitor the level of HCF164 accumulation in the plastid transformants, we used the anti-HCF164 antibody described by Motohashi *et al*. (MOTOHASHI and HISABORI 2006). We noted that the anti-HCF164 antibody cross-reacted with the CCS5 protein (see Figure 2.10C). Interestingly, HCF164 accumulation in *ccs5* plastid transformants expressing HCF164 or $HCF164_{opt}$ is similar (see Figure 2.10C). Therefore, *HCF164* can be expressed in the *ccs5* chloroplast genome and the optimized version of *HCF164* does not improve the accumulation of the corresponding protein. The HCF164-dependent restoration of holocytochrome *f* accumulation in the *ccs5* mutant also confirms that HCF164 must be properly targeted to the thylakoid membrane in *Chlamydomonas*. This suggests that the HCF164 targeting sequence must be recognized by *Chlamydomonas* import machinery to correctly address the protein to the thylakoid membrane. Moreover, the HCF164-dependent restoration of holocytochrome *f* assembly indicates that the redox active domain of HCF164 is facing

the lumenal side of the thylakoid membrane in the *ccs5* mutant. From these results, we can conclude that HCF164 is the functional homolog of CCS5 and also confirm that the site of action of CCS5 is as expected in the thylakoid lumen.

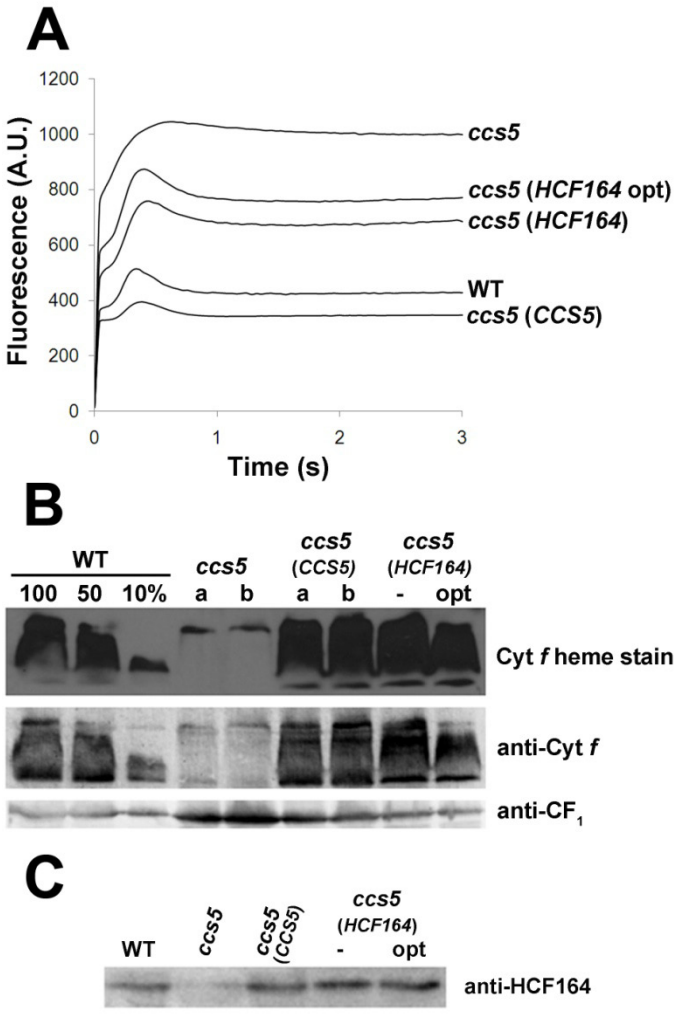


Figure 2.10. Heterologous functional complementation of *C.reinhardtii ccs5* mutant by *A. thaliana HCF164* expressed in the chloroplast genome.

For ABC, the *ccs5* mutant (*T78.15b*$^{-}$) was transformed by electroporation with the Pm^{R} cassette (*ccs5*), with the wild type *Chlamydomonas CCS5* gene (*CCS5*) and Pm^{R} cassette (nuclear transformation). The *ccs5* mutant was co-transformed by biolistic with pmp

continued

Figure 2.10. continued.
HCF164 (HCF164) or pmp *HCF164* codon optimized (HCF164 opt) and p699-aadA (chloroplast transformation).

A. Fluorescence kinetics indicate the restoration of the cytochorme b_6f complex in the complemented *ccs5* mutant. Fluorescence induction and decay kinetics of the *ccs5*, *ccs5* complemented by *HCF164* optimized (HCF164 opt), *ccs5* complemented by wild type *HCF164* (HCF164), wild type (WT) and *ccs5* complemented by wild type *CCS5* (*CCS5*) are shown with one representative transformant. For detailed informations, see Figure 2.2C.

B. Holocytochrome *f* accumulation is restored in the *ccs5* mutant transformed by either wild type *CCS5* or wild type *HCF164* (*HCF164*) or its codon optimized version (*HCF164opt*). a and b refer to two independent transformants.

C. Immunodetection performed by using the anti-HCF164 antibody (MOTOHASHI and HISABORI 2006).
For B and C, experimental conditions are the same as described in Figure 2.5C.

2.5. Discussion

In this study, we report the identification of the *Chlamydomonas reinhardtii ccs5* mutant that displays a specific block in the assembly of cytochrome *f* and c_6, two plastid *c*-type cytochromes. We show that 1) the *ccs5* mutant can be chemically rescued by exogenous thiols, 2) the *CCS5* gene encodes a thioredoxin-like protein with similarity to *Arabidopsis* HCF164, a protein involved in the assembly of the cytochrome b_6f complex and that the CCS5 protein 3) displays disulfide reductase activity, 4) interacts with apocytochrome *f*, c_6 and the newly identified apocytochrome c_{6A}, 5) is able to reduce an intramolecular disulfide at the C*XX*CH motif in the apocytochrome *f* target and 6) the *ccs5* mutant can be complemented by the *Arabidopsis* HCF164 ortholog when expressed in the chloroplast genome.

CCS5/HCF164 is a CXXCH disulfide reductase involved in plastid c-type cytochrome assembly

Through this work, we have identified CCS5, the algal ortholog of *Arabidopsis* HCF164 that was previously shown to be required for cytochrome b_6f assembly in *Arabidopsis* (LENNARTZ *et al.* 2001; MOTOHASHI and HISABORI 2006). *Chlamydomonas* CCS5 and *Arabidopsis* HCF164 are similar proteins (35% identity) and it is expected that the algal protein also localizes to the thylakoid membrane with the redox domain facing the lumen similarly to its vascular plant counterpart (see Figure 2.4A). The heterologous functional complementation of *Chlamydomonas ccs5* mutant by *Arabidopsis* HCF164 ortholog expressed in the chloroplast genome confirms that HCF164 is the functional homolog of CCS5 and a chloroplast site of action for the algal protein (see Figure 2.10). CCS5/HCF164 is very well conserved from unicellular algae to vascular plants. Remarkably, the WC*XX*C redox motif is strictly conserved in all orthologs (see Figure 2.4B). CCS5/HCF164 displays some similarity to *Synechococcus* sp. TxlA that was shown to be required for photosynthesis. However, it is not clear if TxlA is the cyanobacterial counterpart of plastid CCS5/HCF164 because the photosynthetic defects due to mutations in the *txlA* gene were not examined in detail (COLLIER and GROSSMAN 1995).

The implication of *Arabidopsis* HCF164 in a thiol reducing pathway for cytochrome *c* assembly was not ascertained because a specific defect in the maturation of holocytochrome *f* could not be demonstrated (LENNARTZ *et al.* 2001). Note that in land plants like *Arabidopis*, cytochrome *f* is the only *c*-type cytochrome that is required for

photosynthesis. Cytochrome c_6, the substitute for plastocyanin in copper deficient conditions is not present in land plants but only in green algae and cyanobacteria (BIALEK *et al.* 2008; KERFELD and KROGMANN 1998). Based on the fact that a) the *ccs5* mutant displays a dual deficiency in the assembly of plastid cytochromes *c*, namely cytochrome *f* and cytochrome c_6 and b) HCF164 can restore holocytochrome *f* assembly when expressed in *ccs5*, we have demonstrated the placement of CCS5/HCF164 in the cytochrome *c* assembly pathway (see Figures 2.1, 2.2, 2.5 and 2.10). The DTT dependent rescue of the *ccs5* mutant indicates that the CCS5/HCF164 protein acts as a reductant in the heme attachment reaction to the C*XX*CH motif (see Figure 2.3). Indeed, recombinant CCS5 displays a disulfide reductase activity in the insulin assay, an indication that disulfides are the targets of CCS5 activity *in vivo* (see Figure 2.6). Moreover we have shown that apocytochromes *f*, c_6 and c_{6A} can physically interact with CCS5 and that apocytochrome *f* heme binding site can be acted upon by CCS5 and converted from oxidized to reduced form (see Figures 2.7, 2.8 and 2.9). This is the first demonstration that a cytochrome *c* assembly factor involved in redox chemistry carries this biochemical activity. The view that CCS5/HCF164 is only active as an apocytochrome disulfide reductase, may be oversimplified and it is also conceivable that the protein exerts some chaperoning activity on its apocytochrome *c* targets. Recent work on CcmG, the CCS5/HCF164 counterpart in bacterial System I points to an apocytochrome *c* chaperone function that is distinct from the redox activity (TURKARSLAN *et al.* 2008). Such function could be required to present the reduced apocytochrome *c* for efficient ligation to the heme co-factor. The structure of ResA, the bacterial system II equivalent of

CCS5/HCF164 shows that some residues could serve as a binding interface for apocytochrome *c* substrates when the protein is in the reduced conformation (COLBERT *et al.* 2006; CROW *et al.* 2004). The fact that the cysteines in CCS5 are not strictly required for interaction with its apocytochrome *c* targets in a yeast two-hybrid assay is compatible with a possible chaperone function for this assembly factor (see Figure 2.7).

Both heme and the cysteine sulfhydryls need to be reduced for stereospecific attachment of heme to the apocytochromes *c* (FERGUSON *et al.*; GIEGE *et al.* 2008; HAMEL *et al.* 2009; KRANZ *et al.* 2009). It is unlikely that CCS5 is also involved in the reduction of heme based on the fact that disulfide/dithiol exchange is a two-electron process while hemes can only receive one electron at a time. However this possibility cannot be formally excluded if two hemes are being reduced at the same time or if another electron acceptor is involved.

What are other candidate targets of CCS5/HCF164 activity in the thylakoid lumen? Thiol-trapping experiments using *Arabidopsis* HCF164 that carries a mutation at the second cysteine in the thioredoxin motif have identified cytochrome *f* as a target of *Arabidopsis* HCF164 (MOTOHASHI and HISABORI 2006). This is consistent with our finding that cytochrome *f* assembly is deficient in the *ccs5* mutant and that the disulfide in apocytochrome *f* heme binding site is the target of CCS5 reducing activity. Based on the fact that *ccs5*/*hcf164* mutants display a specific defect in cytochrome b_6f (data not shown, LENNARTZ *et al.* 2001), it is expected that the relevant targets of CCS5/HCF164 control b_6f biogenesis and/or activity. Interestingly, thiol-trapping experiments did not allow the identification of cytochrome c_{6A} as a target of HCF164 (LENNARTZ *et al.* 2001).

This is expected considering that cytochrome c_{6A} is poorly expressed in *Arabidopsis* (GUPTA *et al.* 2002a). Note that we failed to detect cytochrome c_{6A} by heme stain, in wild-type *Chlamydomonas* protein extracts (data not shown), an indication that algal cytochrome c_{6A} is also a low abundance molecule. However, from our yeast two-hybrid experiment, it is likely that apocytochrome c_{6A} requires HCF164/CCS5 for conversion to its holoform.

An additional target identified by thiol trapping is the Rieske protein, a structural subunit of the cytochrome b_6f complex (MOTOHASHI and HISABORI 2006). The relevance of this interaction is unclear but it is possible that the disulfide bond in the Rieske protein (CARRELL *et al.* 1997) needs to be reduced under certain conditions to regulate the activity of the cytochrome b_6f complex. The functionality of this disulfide in the b_6f complex remains unexplored. One possible additional target is the STT7/STN7 kinase which interacts with cytochrome b_6f and is required for state transitions (DEPÈGE *et al.* 2003; LEMEILLE *et al.* 2009), an adaptation of the photosynthetic machinery to changes in light intensities that involves the cytochrome b_6f complex (LEMEILLE *et al.* 2009). STT7/STN7 is a transmembrane thylakoid protein with a conserved pair of cysteines exposed to the lumen. Alteration of the conserved cysteines inactivates the kinase activity suggesting that the disulfide bonded form of STT7/STN7 is the active form (LEMEILLE *et al.* 2009). Because state transitions are a reversible process, it is possible that CCS5/HCF164 mediates the reduction of the disulfide in redox situation where the kinase requires to be inactive (LEMEILLE *et al.* 2009). PS1-N, a structural subunit of photosystem I was also identified in the thiol-trapping experiments and shown to be acted upon in an

in vitro assay by HCF164 (MOTOHASHI and HISABORI 2006). The significance of this finding is obscure because loss of HCF164 does not seem to impact photosystem I function (LENNARTZ *et al.* 2001). However, it is possible to envision that PSI-N undergoes thiol-disulfide regulation under certain conditions and that CCS5/HCF164 acts as the transducer of reducing power. Other cysteine containing proteins in the lumen such as violaxanthin de-epoxidase might also be regulated by CCS5/HCF164 reducing activity. Violaxanthin de-epoxidase is an enzyme involved in photoprotection whose activity is inhibited under reducing conditions (BILGER and BJÖRKMAN 1990; NIYOGI *et al.* 1998).

Operation of a multi-component trans-thylakoid thiol reduction pathway

If CCS5/HCF164 acts as a reductant in the thylakoid lumen, where does the reducing power come from? By analogy to the bacterial membrane, a transmembrane thiol-disulfide relay from stroma to lumen must operate at the thylakoid membrane. It is very likely that CCDA in the plastid is involved in such a pathway (NAKAMOTO *et al.* 2000; PAGE *et al.* 2004). The cytochrome b_6f deficient phenotype due to loss-of-function mutations in the *CCDA* gene is compatible with such a proposed activity but does not indicate if 1) the cytochrome b_6f deficient phenotype is due to loss of cytochrome *f* assembly and 2) redox chemistry is required in the context of plastid cytochrome *c* assembly *in vivo*. *Chlamydomonas* remains the best model system to confirm the placement of CCDA in the cytochrome *c* assembly pathway because of the availability of *ccs* mutants that are specifically blocked in the conversion of plastid apocytochromes *c* to their corresponding holoforms (DREYFUSS and MERCHANT 1999; HOWE and MERCHANT

1992; PAGE *et al.* 2004; XIE *et al.* 1998). However, no *CCS* locus was found to be affected for the *CCDA* gene in any of the genetically defined *CCS* loci (PAGE *et al.* 2004). Hence, the involvement of CCDA in cytochrome *c* assembly, although likely, is still hypothetical at this point. The reductant of CCDA on the stromal side is also not known but stromal thioredoxins are possible electron donors (NAKAMOTO *et al.* 2000). Recombinant spinach thioredoxin-*m* was shown to be able to reduce CCDA and HCF164 in an *in organello* assay, an indication that it might be recruited as a reductant on the stroma to convey redox power across the thylakoid membrane (MOTOHASHI and HISABORI 2006; MOTOHASHI and HISABORI 2010). The fact that the complete absence of CCS5/HCF164 does not abolish *c*-type cytochrome assembly suggests that there is functional redundancy for the provision of reducing equivalents to apocytochrome *f* and c_6 (see Figure 2.1) (LENNARTZ *et al.* 2001). It is likely that one of the genetically defined *CCS* loci is functionally redundant with CCS5/HCF164. We hypothesize that the *CCS4* gene product is functionally redundant with CCS5/HCF164 based on the fact that the *ccs4* mutant is not completely deficient in holocytochrome *f* (XIE *et al.* 1998) and is also rescued by exogenous thiols (PAGE *et al.* 2004). The *CCS4* locus must encode a novel redox factor because it does not correspond to the *CCDA* gene (PAGE *et al.* 2004).

CHAPTER 3

CCS4, A NOVEL COMPONENT OF THE DISULFIDE-REDUCING PATHWAY REQUIRED FOR CYTOCHROME C ASSEMBLY IN PLASTIDS

3.1. 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 C*XX*CH 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 c_6. 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.

3.2. 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 sulfhydryls in the apocytochrome *c* (THÖNY-MEYER 1997). The cysteine sulfhydryls are found in a C*XX*CH motif, also referred to as the heme binding site, where histidine acts as one of the axial ligands of heme. C*XX*CK, C*XXX*CH or C*XXXX*CH 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/A*XX*CH 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 C*XX*CH in apocytochrome *c* prior to the heme ligation (ALLEN *et al.*

2003a; 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* C*XX*CH 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 *c*ytochrome *c* *s*ynthesis) (HOWE and MERCHANT 1992; HOWE *et al.* 1995; XIE *et al.* 1998). These mutants are deficient for membrane-bound cytochrome *f* and 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 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 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 (GABILLY *et al.* 2010; LENNARTZ *et al.* 2001). The recombinant form of CCS5/HCF164 can reduce a disulfide at the C*XX*CH 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.

3.3. Experimental procedures

3.3.1. 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 (*cw*$_{15}$ *nit1 mt*$^-$) 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/m^2/s) for *ccs4* and *ccs5* strains or under standard illumination for wild type strains (300 μmol/m^2/s) as described in (HOWE and MERCHANT 1992). Copper deficient media are used to induce the expression of cytochrome c_6 (QUINN and MERCHANT 1998).

3.3.2. 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 (300μmol/m^2/s). An eight kb *BamH*I and a one kb *Sac*II fragments 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 Pccs4-ORF2-NdeI (5'-AACCCATATGTCGACTGGCATTGAGG-3') and L-Pccs4-ORF2-XbaI (5'-AACT CTAGATCACTTGGTTGCCTGC-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-EcoRI (5'-CGATAAGCTTGATATC GAATTCATGGCTATTTCAAAAGGCATTGAGG-3') and PORF5-R-XbaI (5'-GGTC CAGCTGCTGCCATCTAGATCACTGGTTGCCTGCTCCTGG-3') as primers and pSK-CCS4 *Sac*II as a template. The final construct is pSL18-CCS4 (ORF2).

3.3.3. 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'CGGAATTCTCACGAGGGCACCAGGCGCG-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.

3.3.4. 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 *Sac*II (*ccs4-Sac*) or with pCB412 and pSK-CCS4 *Bam*HI (*ccs4-Bam*) were grown in TAP medium at 25°C with 25 μmol/m^2/s of light. At about 6 x 10^6 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'-GCTTCCTCCCTGCAGCCGTCCT-3' and 5'-GCGGGATCAAGCAGCGACAAGT-3' for *CCS1*; 5'-TGGTTGCCTGCT CCTTGGAC-3' and 5'-GCACGGGCTCAGATGAATGG-3' for *CCS4*: 5'-GCGGGG TCGAGAGGTTATGG-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) was calculated as followed: RTL = 1000 x [mean PCR efficiency for $CBLP^{\mathrm{Ct}CBLP}$ x [mean PCR efficiency for *gi*] $^{\mathrm{Ct}gi}$.

3.3.5. 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.

3.4. Results

3.4.1. 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 controls 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).

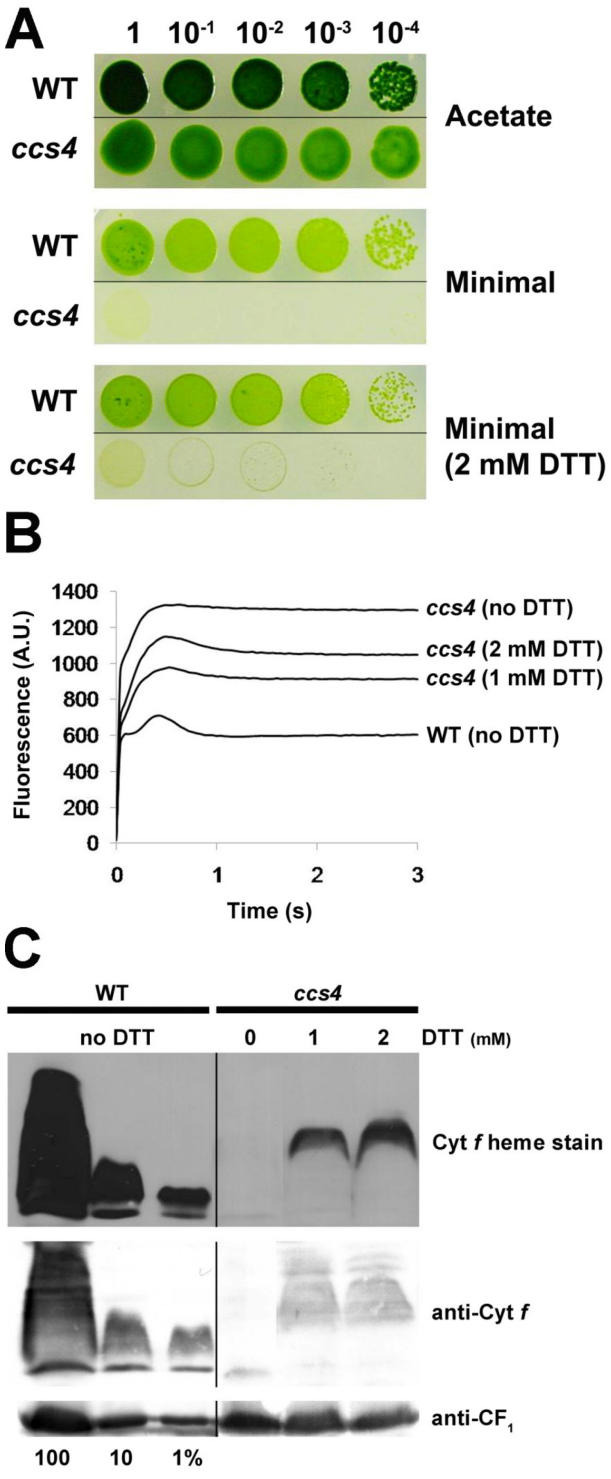


Figure 3.1. The *ccs4* mutant is partially rescued by exogenous thiols.

A. DTT dependent photosynthetic rescue of *ccs4*.

continued

Figure 3.1. continued.
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/m^2/s of light. Cells grown phototrophically with or without DTT were incubated at 25°C for 14 days with 250 µmol/m^2/s 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 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 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 cytochrome 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 (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 CF_1 of the ATPase that serves as a loading control. Dilutions of the wild type sample serve to estimate the cytochrome *f* abundance.

As shown in Figure 3.1A, 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 (see Figure 3.1B). Heme stain and immunoblot

analysis confirmed that levels of holocytochrome *f* are increased in DTT treated cells (see Figure 3.1C). 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.

3.4.2. 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 *Sac*II fragment suggesting that the *CCS4* gene is very small (see Figure 3.2A). This one kb fragment restored photosynthetic growth (see Figure 3.2A), fluorescence rise and decay kinetics, indicating that the cytochrome b_6f complex is functional (see Figure 3.2B), and the accumulation of holoforms of cytochrome *f* and c_6 to wild type levels (see Figure 3.2C).

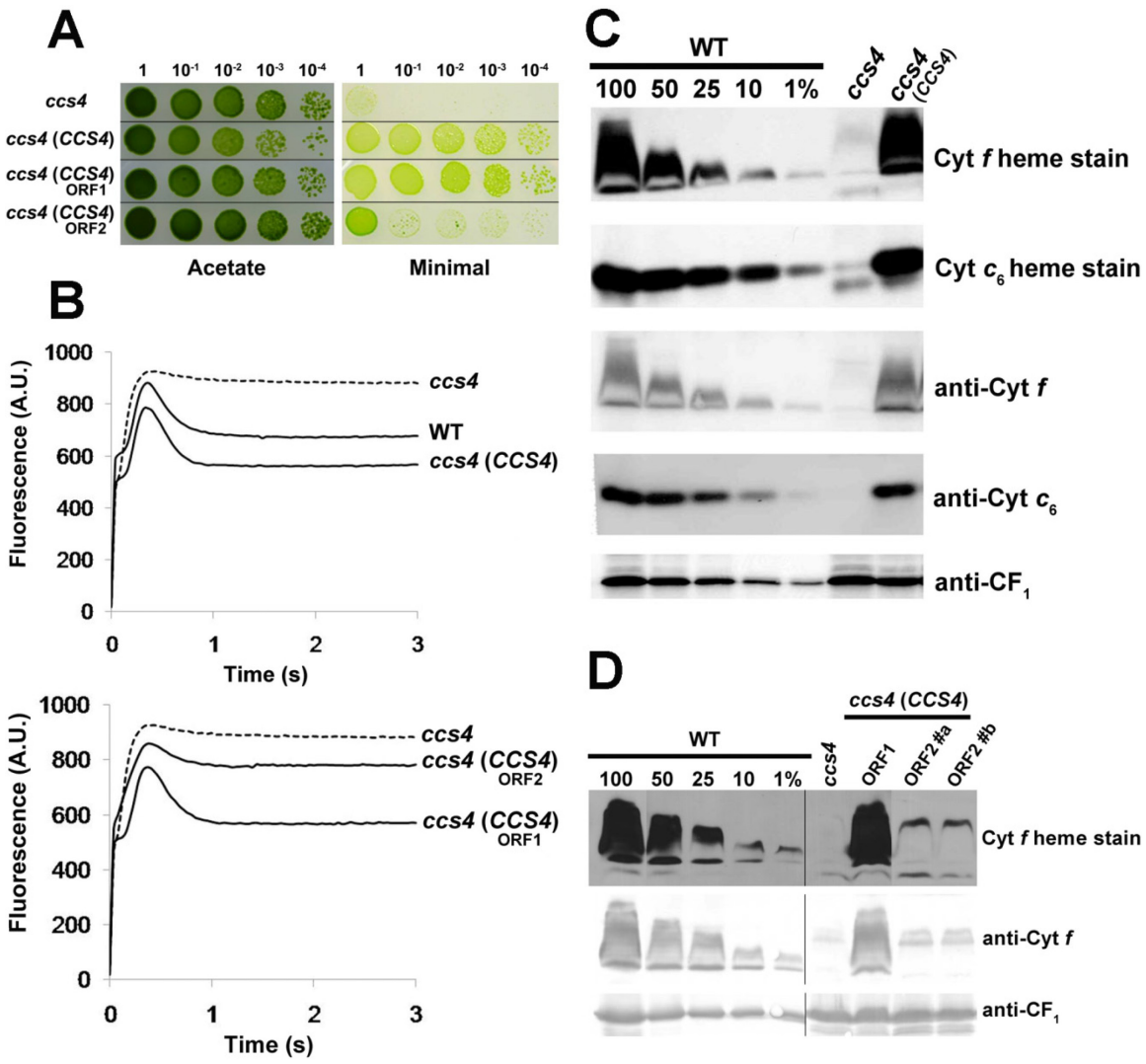


Figure 3.2. Complementation of the *ccs4* mutant.

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-*Sac*II 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.*

continued

Figure 3.2. continued.
Ten-fold dilution series of each transformant were plated on Acetate (heterotrophic conditions, 20 μmol/m^2/s of light) and Minimal medium (phototrophic conditions, 250 μmol/m^2/s of light) and incubated at 25°C for 1 week and 3 weeks, respectively.

B. Fluorescence kinetics indicates restoration of cytochrome b_6f 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.

3.4.3. The *CCS4* gene encodes a unique protein with no known motif

RT-PCR experiments showed that the genomic region corresponding to the one kb *Sac*II 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).

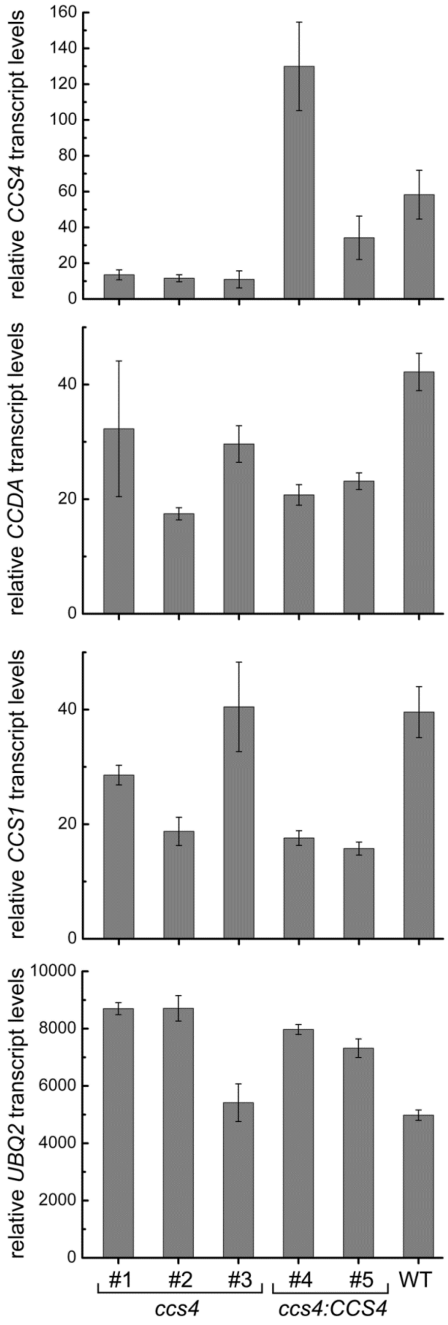


Figure 3.3. Relative *CCS4*, *CCDA* and *CCS1* mRNA abundance in *ccs4* and *ccs4* (*CCS4*) complemented strains.

continued

Figure 3.3. continued.
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 *Bam*HI (#4) or co-transformed by pCB412 and pSK-CCS4 *Sac*II (#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.

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 (see Figure 3.3). One ORF was identified from sequencing of the RT-PCR products (see Figure 3.4; 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 (see Figure 3.4), 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 *Sac*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 non-sense mutation at residue Q_{50} in the predicted sequence (CAG to TAG) and presumably produces a non-functional truncated protein (see Figure 3.4). 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. Out of 22 Pm^R transformants, 12 were able to grow photosynthetically and displayed wild type fluorescence rise and decay kinetics (see Figure 3.2AB). As expected from the restoration of the photosynthetic growth, cytochrome *f* assembly is also restored to wild type levels (see Figure 3.2D). The level of complementation is identical to that of the transformants carrying the one kb *Sac*II genomic fragment, suggesting that ORF1 encodes the *CCS4* gene product (see Figure 3.2ABD). In order to determine which of the two methionines (M1 and M32) serve as an intiation codon (see Figure 3.4), 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 (see Figure 3.4). We cloned the truncated sequence from ATG to stop (ORF2) in the same expression vector used for our complementation experiments. Out of 103 Pm^R transformants, 43 exhibited partial complementation of the photosynthetic growth defect and pseudo-wild type fluorescence rise and decay kinetics (see Figure 3.2AB). 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 (see Figure 3.2D). 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 (see Figure 3.1C). 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 (see Figure 3.2A). 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.

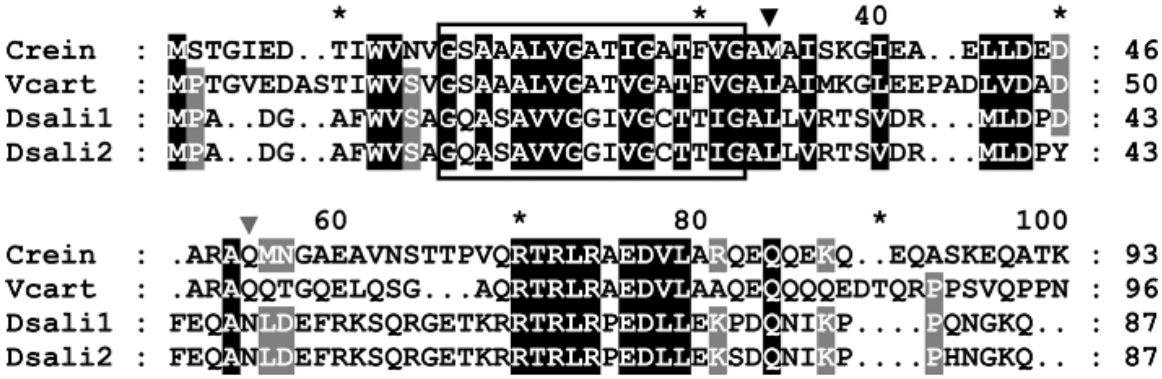


Figure 3.4. Alignment of *Chlamydomonas*, *Volvox* and *Dunaliella* CCS4 proteins.

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.

3.4.4. 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 (see Figure 3.3 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 (see Figure 3.5).

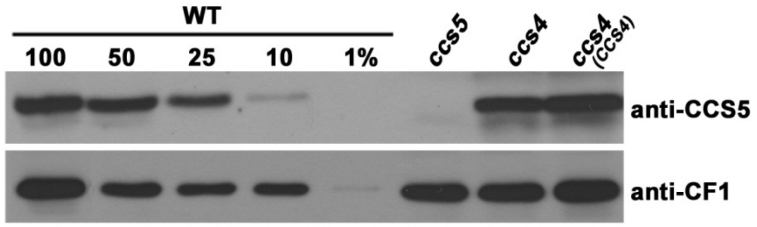


Figure 3.5. Accumulation of the CCS5 protein in *ccs4*.

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 antisera against CCS5 or CF_1 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.

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 (Pm^R), a trait conferred by the selectable marker on the construct. Out of 44 Pm^R transformants, 14 were suppressed for the CCS phenotype based on the partial restoration of the photosynthetic growth defect (see Figure 3.6A). While 12 transformants were weakly suppressed, two displayed an increased level of phototrophic growth (see Figure 3.6A). To confirm our results, we co-transformed 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). Out of 45 Pm^R 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 Pm^R transformants, 12 displayed photosynthetic growth and fluorescence transients indistinguishable from wild type (see Figure 3.6AB). 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 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 (see Figure 3.6B). However, we could only demonstrate enhanced holocytochrome *f* accumulation in the strongly suppressed transformants (see Figure 3.6C).

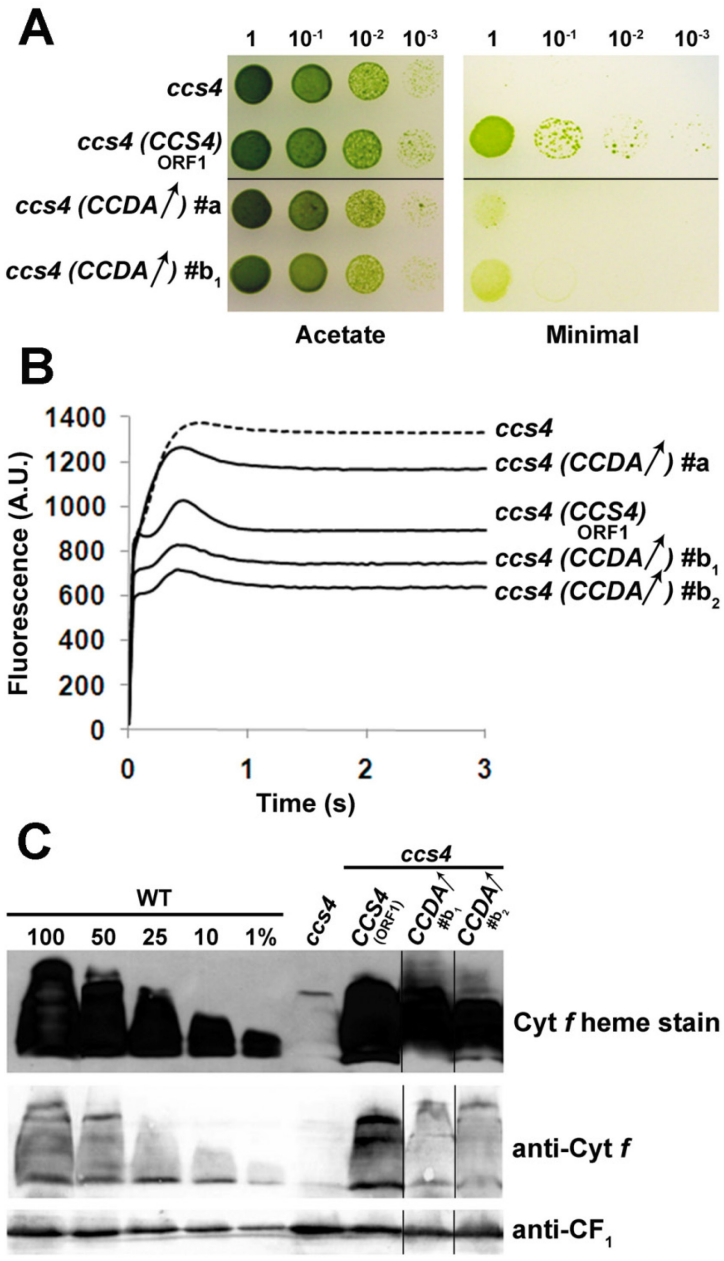


Figure 3.6. Expression of an ectopic copy of the *CCDA* gene partially suppresses *ccs4*.

continued

Figure 3.6. continued.
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 #b_1) or co-transformed with pSL18 and the *CCDA* cDNA cloned (without promoter and terminator sequences) in pBluescript (*CCDA*↑, transformant #b_2).

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/m^2/s of light) and minimal medium (phototrophic conditions, 300 μmol/m^2/s of light) and incubated at 25^{o}C for 1 week and 3 weeks, respectively.

B. Fluorescence kinetics indicate partial restoration of cytochrome b_6f in *ccs4* expressing an ectopic copy of *CCDA*.
Fluorescence induction and decay kinetics were measured as described in Figure 3.1B.

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 3.2C.

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 (see Figure 3.7).

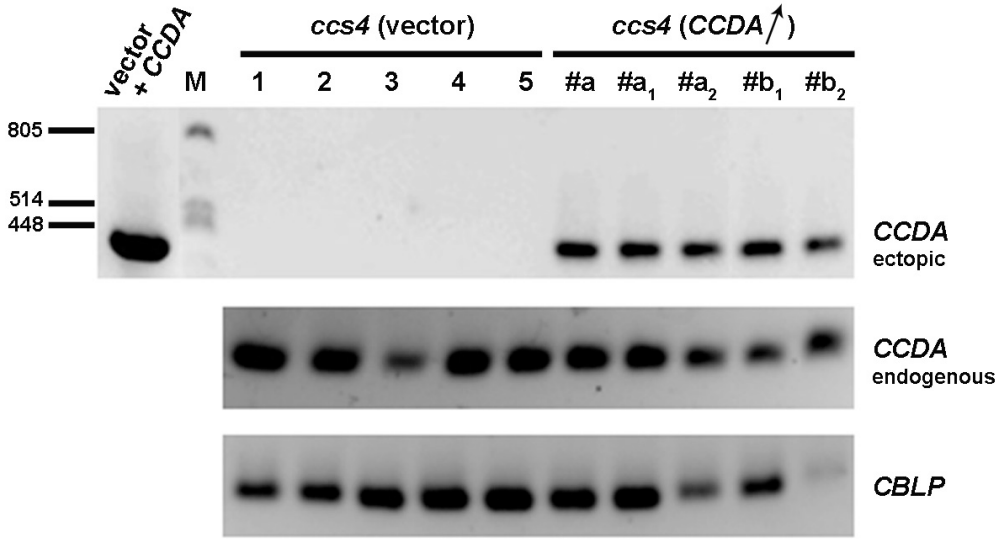


Figure 3.7. Expression of ectopic and endogenous *CCDA* in *ccs4* transformants.

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'-CCCTCGTC AGCCCTCTGTGT-3') to detect the endogenous *CCDA* transcript, PpSL18-1 (5'-CTGCTACTCACAACAAGC-3') and PCCDA1-2 (5'-ACCATTGCCGTTGTCGTCCT GGCTATAC-3') to detect the ectopic *CCDA* transcript and PCBLP-1 (5'-GCCACACC GAGTGGGTGTCGTGCG-3') and PCBLP-2 (5'- CCTTGCCGCCCGAGGCGCACAG CG-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, #a_1, #a_2 and transformants #b_1 and #b_2 show weak and strong suppression of the photosynthetic deficiency, respectively. Vector + *CCDA* corresponds to the pSL18-CCDA construct used to obtain the transformants #a, #a_1, #a_2 and #b_1. Transformant #b_2 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 amplication products were separated by electrophoresis in agarose gel and ethidium bromide stained. The gel was imaged using an imaging system.

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.

3.5. 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* (see Figure 3.1) 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 C*XX*CH 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* C*XX*CH 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 (see Figure 3.6). 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-*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; MOTOHASHI and HISABORI 2010).

What is the 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 (see Figure

3.4). One possibility is that the *ccs4* mutation results in a loss of CCDA function. This is compatible with the fact that 1) *ccs4* can be partially rescued by DTT (see Figure 3.1), as seen in bacterial *ccdA*/*dsbD* mutants that are restored for cytochrome *c* assembly in the presence of exogenous thiols (BECKETT *et al.* 2000; DESHMUKH *et al.* 2000; SAMBONGI and FERGUSON 1994) and 2) expression of *CCDA* can partially by-pass the *ccs4* mutation (see Figure 3.6). 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* (see Figure 3.2). 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* C*XX*CH 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/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 (see Figure 3.4). 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 (see Figure 3.4). 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).

CHAPTER 4

CONCLUSIONS AND PERSPECTIVES

In this work, we report the identification of the first trans-thylakoid disulfide-reducing pathway operating in the plastid lumen and provide evidence that thiol-based redox chemistry is a controlled process in this compartment. This disulfide-reducing pathway was discovered in the context of plastid *c*-type cytochromes assembly in *Chlamydomonas*. While the structures of several cytochromes *c* are known (ALLEN *et al.* 2003a; FERGUSON *et al.* 2008; FULOP *et al.* 2009), the processes by which they are assembled are still far from being completely understood and appear surprisingly complex (BONNARD *et al.* 2010; HAMEL *et al.* 2009; KRANZ *et al.* 2009). One chemical requirement for cytochrome *c* biogenesis is the reduction of the apocytochrome C*XX*CH heme binding motif, prior to the covalent heme ligation. This chemical requirement was first rationalized in the case of bacterial cytochromes *c* because the periplasm is a compartment where oxidative folding of proteins takes place. The requirement for a disulfide-reducing pathway for plastid cytochrome *c* assembly needed to be demonstrated as there was little support for a pathway controlling sulfhydryl oxidation in the thylakoid lumen.

In this work, we show that this biochemical requirement for cytochrome *c* assembly in the plastid is controlled by at least three components CCS5, CCS4 and CCDA postulated to be involved in the delivery of reductants from stroma to lumen across the thylakoid membrane (GABILLY *et al.* 2010; GABILLY *et al.* 2011; LENNARTZ *et al.* 2001; MOTOHASHI and HISABORI 2006; MOTOHASHI and HISABORI 2010; PAGE *et al.* 2004).

CCS5 is a thioredoxin-like protein with a disulfide CXXCH reductase activity

CCS5 is a membrane-anchored lumen-facing, thioredoxin-like protein that shows 35% sequence identity to *Arabidopsis* HCF164, a protein previously identified as being involved in cytochrome b_6f biogenesis (LENNARTZ *et al.* 2001). It is likely that the cytochrome b_6f deficiency of *hcf164* mutant is due to a defect in heme attachment to apocytochrome *f*. Indeed, *Arabidopsis* HCF164 is the functional equivalent of CCS5 as we showed that its expression in the chloroplast genome of *ccs5* can complement the CCS phenotype. The apocytochrome *c* disulfide reductase activity of CCS5 is inferred from the findings that 1) the *ccs5* mutant can be chemically rescued *in vivo* by application of exogenous thiols, 2) CCS5 interacts with plastid cytochromes *c* in a yeast two-hybrid assay and 3) CCS5 has the ability to reduce a disulfide bonded C*XX*CH in apocytochrome *f in vitro* (GABILLY *et al.* 2010).

CCS4 is a novel component of a disulfide-reducing pathway with unknown activity

The novel component identified in this work is CCS4, a 93 amino acid protein with a hydrophobic amino-terminus and a hydrophilic carboxyl-terminal domain with several charged residues. The identification of CCS4 is intriguing because this component does not display any motif suggesting a role in thiol-based redox chemistry. The thiol-

dependent photosynthetic rescue of the *ccs4* mutant and the suppression of the *ccs4* phenotype by ectopic expression of CCDA, the ortholog of bacterial CcdA/DsbD at the thylakoid membrane, confirms the activity of CCS4 in a disulfide-reducing pathway for cytochrome *c* assembly (GABILLY *et al.* 2011). Surprisingly, the protein appears to be conserved exclusively in a subset of green algae such as *Volvox carteri* and *Dunaliella salina*, two close relatives of *Chlamydomonas* in the Volvocales order. No CCS4 orthologs in cyanobacteria or land plants could be identified based on sequence similarity. While it is conceivable that CCS4 is specific to Volvocales, another possibility is that the primary sequence has considerably diverged and CCS4 orthologs cannot be recognized on the basis of sequence similarities. Interestingly, we noted that HCF153, a protein required for photosynthesis in *Arabidopsis* displays all the features of *Chlamydomona*s CCS4 (see Figure 4.1) (LENNARTZ *et al.* 2006). Both proteins display a putative amino-terminus transmembrane domain followed by a hydrophilic carboxyl-terminal domain with several charged residues. Remarkably, the charge distribution is similar in both proteins (9 positive and 12 negative residues for CCS4, 9 positive and 14 negative residues for HCF153). According to the positive-inside rule that governs the topology of bacterial and thylakoid membrane proteins, the C-terminal domain of both proteins is predicted to be exposed to the stromal side of the thylakoid membrane.

Arabidopsis thaliana HCF153 (9+ residues, 14- residues)

LLAFAIPATLIAATVFTSIKIADKLDEDFLEDIALNQAIKAAEKGENGEGDISLDDVIQEPVLQ

RTRNRPKREV

Chlamydomonas reinhardtii CCS4 (9+ residues, 12- residues)

MSTGIEDTIWVNVGSAAALVGATIGATFVGAMAISKGIEAELLDEDARAQMNGAEAVNSTTPVQ

RTRLRAEDVLARQEQQEKQEQASKEQATK

Figure 4.1. HCF153, a candidate *Arabidopsis thaliana* ortholog of *Chlamydomonas reinhardtii* CCS4.

Amino acid sequences from *A. thaliana* HCF153 and *C. reinhardtii* CCS4
The predicted stromal targeting transit peptide is underlined
The putative membrane anchor is boxed
The positive residues and negative residues of the primary sequences are highlighted in blue and red, respectively.
Motifs with conserved or similar residues are highlighted in yellow.

HCF153-like proteins can be detected in other vascular plants and comparison with CCS4-like proteins in Volvocales reveal that sequence similarity is only restricted to two conserved motifs (AD/EK/LLDED and RTRN/LR) (see Figure 4.1). It is likely that these motifs are important for the function of the proteins. The *hcf153* mutant is photosynthetic deficient and displays a high chlorophyll fluorescence phenotype due to a reduced accumulation of the cytochrome b_6f complex (LENNARTZ *et al.* 2006). The photosynthetic defect was attributed to a block in the post-translational step of the biogenesis of the cytochrome b_6f complex based on the finding that major subunits of the cytochrome b_6f complex were transcribed and translated. HCF153 was localized at the thylakoid membrane but there is no topological information as to the orientation of the C-terminal domain. Additional experiments are required to determine if HCF153 is the functional

equivalent of CCS4 but the similarities in protein features and loss-of-function phenotypes are in favor of this hypothesis.

CCDA, the thiol disulfide transporter conveys reducing equivalents essential for plastid cytochrome c assembly

The CCDA-dependent suppression of *ccs4* demonstrates for the first time the placement of the thiol/disulfide transporter in plastid cytochrome *c* maturation. Indeed, earlier studies in *Arabidopsis* supported, but did not establish the requirement of plastid CCDA in the conversion of apo- to holocytochromes *c* (PAGE *et al.* 2004).

One working model for the disulfide bond reducing pathway is that CCS5/HCF164, the thioredoxin-like protein, which interacts with apocytochrome *c* targets in the thylakoid lumen, is maintained in a reduced state via the activity of the thiol/disulfide transporter CCDA. CCDA, a polytopic protein of the thylakoid membrane (MOTOHASHI and HISABORI 2010) may, in turn, be reduced by a soluble stromal thioredoxin whose redox state is controlled by the ferredoxin/thioredoxin system composed of Fd/FTR/Trx (see Figure 4.2 and section 1.4.2.1). Experiments using recombinant spinach thioredoxins and purified thylakoids from *Arabidopsis* support the role of Trx-*m* rather than Trx-*f* as the stromal reductant of CCDA (MOTOHASHI and HISABORI 2010). However, *in vivo* proof of such a reaction remains to be established experimentally.

Other targets of the trans-thylakoid disulfide-reducing pathway?

Operation of the CCS5/HCF164-dependent pathway is needed to reduce disulfides in apocytochrome *c* but it is likely that there are other relevant targets of action (GABILLY *et al.* 2010; GABILLY *et al.* 2011; LENNARTZ *et al.* 2001; PAGE *et al.* 2004). Thiol-trapping

experiments using *Arabidopsis* HCF164 carrying a mutation at the second cysteine of the WC*XX*C motif allowed the identification of additional potential targets of action (MOTOHASHI and HISABORI 2006). The Rieske protein, a structural subunit of the cytochrome b_6f complex and PSI-N, a structural subunit of PSI are examples of HCF164-interacting proteins that were retrieved from the thiol-trapping experiments (MOTOHASHI and HISABORI 2006). The relevance of these interactions is not clear but it is possible that the disulfide bond of either the Rieske protein (CARRELL *et al.* 1997) or PSI-N (MOTOHASHI and HISABORI 2006) need to be reduced under certain conditions to regulate the activity of cytochrome b_6f complex or PSI, respectively. An additional target of the CCS5/HCF164-dependent pathway is the STT7/STN7 kinase, which interacts with cytochrome b_6f complex and is required for state transition (DEPÈGE *et al.* 2003; LEMEILLE *et al.* 2009). STT7/STN7 is a transmembrane thylakoid protein with a conserved pair of cysteines facing the lumen. Mutations of the cysteines inactivate the kinase activity, an indication that STT7/STN7 is active in its oxidized form (LEMEILLE *et al.* 2009). State transitions are a reversible process by which the STT7/STN7 dependent phosphorylation triggers the re-organization of the photosynthetic chain (DEPÈGE *et al.* 2003; LEMEILLE *et al.* 2009; ROCHAIX 2010). It is therefore possible that HCF164 regulates state transition by controlling the redox state of STT7/STN7 but this has not been experimentally tested (LEMEILLE *et al.* 2009).

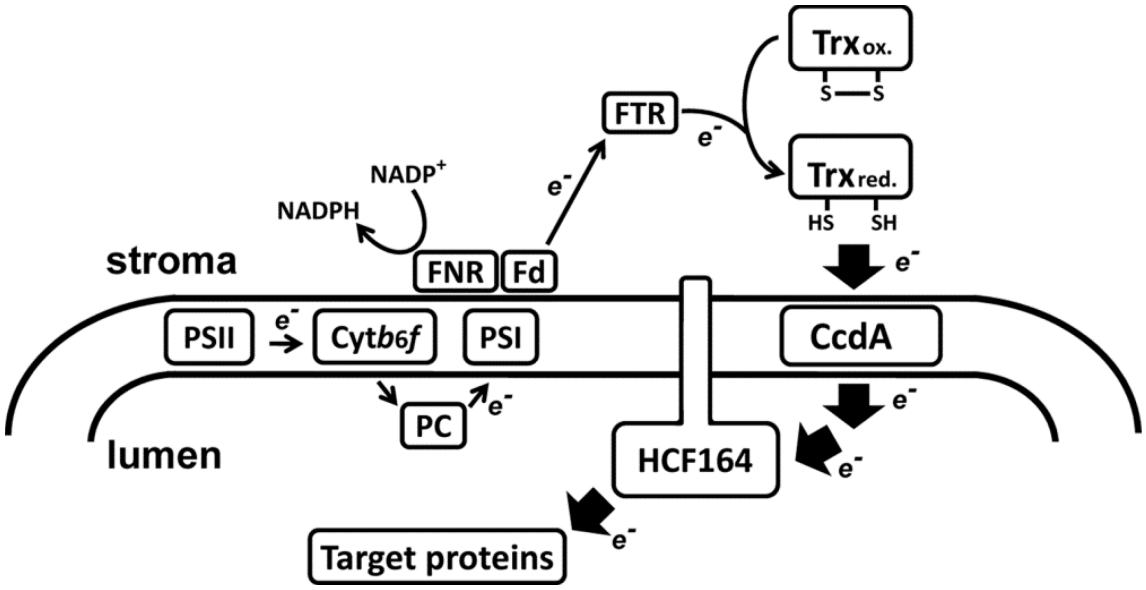


Figure 4.2. Tentative model of a disulfide-reducing pathway which transfers reducing equivalents across thylakoid membranes.

Cytb_6f, cytochrome b_6f complex; e-, reducing equivalents; Fd, ferredoxin; FNR, ferredoxin-NADP+ reductase; FTR, ferredoxin-thioredoxin reductase; PSI, photosystem I; PSII, photosystem II; PC, plastocyanin; Trx, thioredoxin; HCF164, high chlorophyll fluorescence protein 164; CcdA, cytochrome *c* deficiency protein A; CCS4, cytochrome *c* synthesis 4 (not shown). Source: modified from (MOTOHASHI and HISABORI 2010).

Operation of an alternative thio-reducing pathway?

The fact that the complete absence of CCS5/HCF164 does not abolish *c*-type cytochrome assembly indicates functional redundancy for the provision of reducing equivalents to apocytochromes *c* (GABILLY *et al.* 2010; LENNARTZ *et al.* 2001). It is likely that this functional redundancy is not CCDA-dependent based on the fact that a *ccda*-null mutant is also not completely deficient for cytochrome *f* assembly (PAGE *et al.* 2004). This suggests that other thio-reducing pathways may exist to control disulfide reduction in apocytochrome *c* and possibly other targets. Interestingly, the *ccs4 ccs5* mutant displays a synthetic phenotype and is completely deficient in holocytochrome *f*, an indication that

CCS4 and CCS5 are redundant. This functional redundancy suggests that CCS4 might control a different thio-reducing pathway that the CCS5/HCF164 dependent one. In the CCDA-suppressed *ccs4* strain, restoration of cytochrome *c* assembly can be explained by a compensatory effect via the CCDA-dependent pathway due to enhanced expression of the thiol-disulfide transporter. At the present time the placement of CCS4 in such a pathway remains elusive.

Interestingly, unpublished work in our laboratory supports the involvement of other thylakoid transmembrane proteins in the supply of reductants to the thylakoid lumen. RTL (for Redox Transporter-Like) is proposed to be a novel redox transporter at the thylakoid membrane. RTL displays structural relationship to the CcdA/DsbD family and its occurrence is restricted to land plants and green algae (EITINGER *et al.* 2005; LI *et al.* 2010a). Members of this family are predicited to be polytopic membrane proteins and the presence of a bi-partite targeting sequence with a consensus AXA lumen peptidase cleavage site suggests a thylakoid membrane localization of RTL. Accordingly, RTL in rice was localized at the thylakoid membrane (LI *et al.* 2010a). The occurrence of strictly conserved cysteines in the sequence supports a redox activity of these proteins by analogy to the CcdA/DsbD thiol/disulfide transporter. A reducing activity for RTL is inferred from the finding that heterologous expression of the protein in a bacterial *dsbD* mutant confers resistance to exogenous oxidants such as Cu or diamide (unpublished). Lack of DsbD, in bacteria, results in Cu-sensitivity or diamide-induced toxicity (HINIKER *et al.* 2005; RIETSCH *et al.* 1996). Cu or diamide are believed to exert their toxicity by promoting non-native disulfide bond formation in periplasmic and membrane proteins. In

the absence of DsbD, the DsbC disulfide isomerase in the thio-reducing pathway is rendered inactive and unable to re-arrange aberrant disulfide bonds. By analogy to the bacterial compartment, we propose that RTL in plants participates in redox chemistry in the thylakoid lumen. However, the mode of action of RTL seems different from CCDA, because the conserved cysteines of RTL are not strictly essential for its function (unpublished). It is plausible that unlike CcdA/DsbD that transports reducing equivalents via thiol-disulfide exchange reactions, RTL transports a redox molecule across the membrane. Interestingly, the cytochrome *c*-deficient phenotype of a *dsbD* mutant was shown to be suppressed by overexpression of the bacterial CydDC, a glutathione (GSH) ABC-exporter to the periplasm (PITTMAN *et al.* 2005). The interpretation is that increased level of GSH in the periplasm via overexpression of the exporter can substitute for the absence of the thio-reduction pathway in bacteria. The presence of GSH in the bacterial periplasm suggests that this molecule could be an alternative source of reducing power in this compartment (ESER *et al.* 2009; PITTMAN *et al.* 2005). As the periplasm is topologically equivalent to the thylakoid lumen, it is conceivable that a similar transporter operates at the thylakoid membrane and mediates GSH transport to the lumen. The possibility that RTL is a GSH transporter is an attractive possibility (despite the absence of similarities to the ABC-type GSH transporter) but this awaits experimental testing. Loss of RTL function in rice produces a "yellow-striped leave" phenotype associated with necrotic lesions due to an excessive accumulation of reactive oxygen species (ROS) in the yellow sections. The accumulation of ROS is dependent on light and causes damage to PSII, a photosynthetic complex that appears to be sensitive to photo-

oxidative stress. If GSH is the substrate of RTL transporter, it is conceivable that the molecule acts directly by quenching the ROS produced at the level of PSII (OHTSU *et al.* 2010). Loss of the transporter decreases the concentration of GSH in the thylakoid lumen resulting in an increase of ROS responsible for the damage of PSII. The phenotype due to loss of RTL in rice is compatible with its role in transporting a reductant such as GSH. Based on the phenotype due to loss of function in rice, it is clear that RTL defines a distinct reducing pathway from the one defined by CCDA and CCS5/HCF164.

Operation of a thio-oxidizing pathway?

The evidence that a thio-reducing pathway operates in the thylakoid lumen warrants further experimental investigation of sulfhydryl oxidation as a catalyzed process in this compartment. Parallel investigation in our laboratory led to the discovery of LTO1 (Lumen Thiol Oxidoreductase 1), a polytopic thylakoid membrane protein active as a disulfide bond forming catalyst in the lumen. Loss of LTO1 function in *Arabidopsis* impairs photosynthesis due to a defect in PSII assembly. PsbO, a subunit of PSII oxygen-evolving complex (OEC) was shown to be a relevant target of action of LTO1 sulfhydryl activity. The defect in PSII assembly was attributed to the lack of sulfhydryl oxidation in PsbO due to loss of LTO1 function (KARAMOKO *et al.* 2011).

In bacteria, the thio-reducing pathway composed of the thiol-disulfide transporter CcdA/DsbD or the thioredoxin-like protein, CcmG in system I, ResA/CcsX in system II functions to counteract the thio-oxidizing pathway composed of DsbAB or BdbBC (see sections 1.2.1 and 1.2.2) (DESHMUKH *et al.* 2000; ERLENDSSON and HEDERSTEDT 2002; TURKARSLAN *et al.* 2008). The operation of both pathways in the thylakoid lumen raises

the question if, similarly to bacteria, the trans-thylakoid thio-reducing pathway only functions to counteract the thio-oxidizing pathway.

A disulfide isomerase activity in the lumen?

The operation of a disulfide isomerase activity in the lumen is likely but its molecular identity is currently unknown. Interestingly, violaxanthin de-epoxidase (VDE), an enzyme involved in dissipating excess light contains lumen facing cysteine residues and is active in the oxidized form (BUGOS *et al.* 1998; SOKOLOVE and MARSHO 1976; YAMAMOTO and KAMITE 1972). The activity of VDE must be dependent upon catalyzed disulfide bond formation and isomerization based on the fact that the protein contains 13 cysteines, 8 of which are engaged in disulfide bond while 5 remain reduced (HALL *et al.* 2010). A disulfide isomerase activity for LTO1 is plausible based on the presence of thioredoxin-like domain typical of oxidoreductases of the Protein Disulfide Isomerase (PDI) family (BUCHANAN and BALMER 2005; HATAHET and RUDDOCK 2009; REN and BARDWELL 2011). This family is exemplified by PDI, an enzyme in the endoplasmic reticulum that displays sulfhydryl oxidation, disulfide isomerization and chaperone activity (HATAHET and RUDDOCK 2009). This possibility that LTO1 acts as a disulfide isomerase and/or chaperone similar to PDI awaits experimental validation.

The identification of distinct trans-thylakoid thio-reducing and thio-oxidizing pathways reveals that catalyzed thiol-disulfide chemisty is not restricted to the bacterial periplasm but also operates in the thylakoid lumen. Further genetic and biochemical studies are required to establish how these processes control the biogenesis of the thylakoid compartment and regulate photosynthesis.

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