DISSERTATION

IRON SULFUR CLUSTER BIOGENESIS IN CHLOROPLASTS

Submitted by

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In partial fulfillment of the requirements

For the degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

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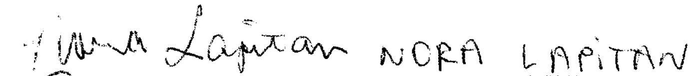

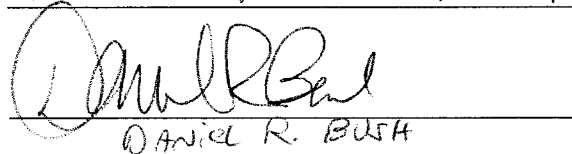

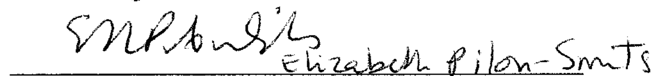

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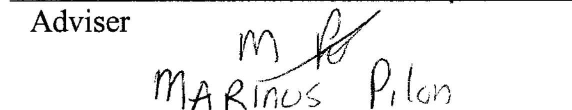

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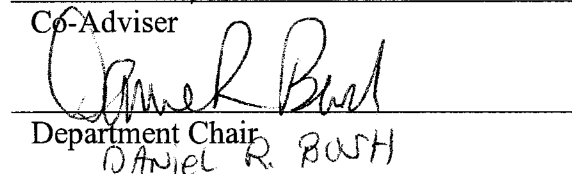

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ABSTRACT OF DISSERTATION

IRON SULFUR CLUSTER BIOGENESIS IN CHLOROPLASTS

Iron-sulfur ([Fe-S]) clusters are essential cofactors for proteins involved in many essential processes. The plant chloroplast is known to have its own biosynthetic machinery for [Fe-S] clusters, but the components have long been unknown. Important processes that depend on this machinery include photosynthesis and nitrate- and sulfate assimilation. The goal of this thesis research was to discover some of the mechanisms and components of this machinery. CpNifS, a NifS-like cysteine desulfurase in chloroplasts, was the first identified component of the [Fe-S] biogenesis machinery in plastids. As described in this thesis, CpNifS was found to be required for chloroplast-mediated [Fe-S] assembly. The removal of CpNifS from chloroplast stroma led to a complete loss of [Fe-S] formation (see Chapter 2). CpSufE is a newly identified component of this machinery (Chapter 3). It activates cysteine desulfurase activity 40-fold and stimulates [Fe-S] formation 20-fold by forming a complex with CpNifS. CpIscA is a recently identified molecular scaffold in this machinery, modulating the formation of a [2Fe-2S] cluster and delivering it to ferredoxin (see Chapter 4). All three proteins AtCpNifS, AtCpSufE and AtCpIscA appear to be present in a ~600 kDa complex in vivo, tentatively named the plastidic [Fe-S] synthase complex in this thesis. These new and other published data were integrated to

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develop a working model for [Fe-S] cluster biogenesis in chloroplasts. Based on *in vivo* expression analyses and preliminary results from transgenic plants (see Chapter 5), implications of the [Fe-S] cluster biogenesis machinery in plastidic homeostasis of iron and sulfur and plant selenium metabolism are discussed.

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Chapter 1

INTRODUCTION

This work was performed to gain insight into mechanisms of iron-sulfur cluster biosynthesis in plant chloroplasts. The topics in this Introduction chapter are presented to provide background information for the research presented in the following chapters.

Overview of [Fe-S] clusters

The iron-sulfur ([Fe-S]) cluster is one of several ancient prosthetic groups, consisting of iron and sulfur atoms in different numbers. For a review of the architecture and kinds of [Fe-S] clusters, see Beinert and coworkers (1997, 2000). As a cofactor, the [Fe-S] cluster is usually bound to a polypeptide by covalent bonds between iron atoms of the [Fe-S] cluster and sulfur atoms of the polypeptide cysteines. However, in the Rieske-type [2Fe-2S] protein, iron atoms of [Fe-S] cluster are coordinated to two histidines and two cysteines. In chloroplasts, the common cluster types include [2Fe-2S], Rieske-type [2Fe-2S], [3Fe-4S], [4Fe-4S], and siroheme-[4Fe-4S], in which the [4Fe-4S] is covalently bound to a siroheme.

Since the iron atom in the cluster can easily gain or lose an electron, thus switching between the redox states Fe^{2+} and Fe^{3+}, the [Fe-S] cluster is capable of transferring electrons in electron transfer chains (for photosynthesis and respiration), and catalyzing redox reactions. Additionally, iron-sulfur clusters act as catalytic centers, regulators of gene expression, and sensors of iron and oxygen (for a review

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see Beinert and Kiley 1999). As more and more proteins are found to be [Fe-S] proteins, the investigations to understand the mechanism of [Fe-S] cluster biogenesis have become an active field of research in recent years.

[Fe-S] cluster assembly systems in organisms

The identification of the NIF gene cluster (Jacobson et al. 1989) and the characterization of NifS as a cysteine desulfurase (Zheng et al. 1993) in the nitrogen-fixing microbe *Azotobacter vinelandii,* marked the beginning of our understanding of [Fe-S] cluster biosynthesis. To date, [Fe-S] biogenesis has been extensively studied in organisms from prokaryotes to eukaryotes, including bacteria (for reviews see Mihara and Esaki 2002a, Johnson et al. 2005), yeast (for review see Lill and Muhlenhoff 2005), plants (for reviews see Pilon et al. 2005, Balk and Lobreaux 2005) and humans (for review see Rouault and Tong 2005). In bacteria, three machineries have been found to assemble [Fe-S] clusters, each of which is encoded by a gene cluster.

The first identified bacterial [Fe-S] cluster biogenesis machinery is the NIF (Nitrogen Fixation) system, found in the nitrogen-fixing microbe *Azotobacter vinelandii* (Jacobson et al. 1998). It is named so because the NIF system assembles [Fe-S] clusters for maturation of nitrogenase in this organism, which is involved in nitrogen fixation. The *nif* gene cluster contains the genes *iscA^nif^-nifU-nifS-nifV-cysE1*. Biochemical and genetic analysis has revealed the function of each gene product. NifS is a cysteine desulfurase, converting cysteine into alanine and sulfide, for assembly of sulfur into the [Fe-S] cluster (Zheng et al. 1993, Zheng and Dean 1994). NifU is a modular homodimeric scaffold protein where a transient [2Fe-2S] cluster is assembled

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(Fu et al. 1994, Agar et al. 2000a, Yuvaniyama et al. 2000) The $IscA^{nif}$ is an alternative scaffold to NifU for mediating nif-specific [Fe-S] cluster assembly (Krebs et al. 2001). As key components of the NIF system, NifS and NifU are required for nitrogenase maturation, but cannot be used for maturation of other [Fe-S] proteins, such as aconitase (Johnson et al. 2005). The NifV protein in the system is a homocitrate synthase (Zheng et al. 1997), likely providing homocitrate for the biosynthesis of the FeMo cofactor of nitrogenase. But it is unknown what the relationship is between the NifV and other NIF proteins in cluster assembly. The cysE1 protein in the NIF system is a serine acetyl transferase important for cysteine synthesis (Evans et al. 1991). The likely role of the cysE-like gene product is to increase the cysteine pool needed for iron-sulfur cluster formation (Zheng et al. 1998).

The second identified bacterial machinery involved in [Fe-S] cluster biogenesis is the ISC (Iron Sulfur Cluster) system, found in both *Azotobacter vinelandii* (Zheng et al. 1998) and *Escherichia coli* (Takahashi and Nakamura 1999). It is the housekeeping machinery producing [Fe-S] clusters for maturation of general [Fe-S] proteins. The isc gene cluster contains the genes *iscRSUA-hscBA-fdx*. IscS is a cysteine desulfurase (Zheng et al. 1998, Mihara et al. 2000, Schwartz et al. 2000). IscU is a scaffold (Agar et al. 2000b, Mansy et al. 2002). IscS binds to IscU and directly transfers sulfur for the assembly of a [Fe-S] cluster on the scaffold (Smith et al. 2001, Urbina et al. 2001, Kato et al. 2002). The hscA and hscB (heat shock cognate) gene products are molecular chaperones (Hoff et al. 2000). They bind to and form a complex with the IscU scaffold, playing a role in the assembly, stabilization, or transfer of [Fe-S] clusters formed on IscU (Silberg et al. 2001). IscA was proposed as

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an alternative scaffold where a transient [2Fe-2S] cluster is formed and transferred to ferredoxin (Ollagnier-de-Choudens et al. 2001) or biotin synthase (Ollagnier-de-Choudens et al. 2004). However, more recent studies supported a role of IscA as an iron-binding protein, which subsequently donates iron for the [Fe-S] cluster assembly on IscU (Ding et al. 2004a, b, Ding et al. 2005). IscR is a repressor of the iscRSUA operon, which is activated by binding a [2Fe-2S] cluster and represses the transcription of the isc operon (Schwartz et al. 2001). Ferredoxin, the *fdx* gene product, is a [2Fe-2S] protein. It is unknown what its role in the ISC machinery is.

The third identified bacterial machinery involved in [Fe-S] cluster biogenesis is the SUF (mobilization of Sulfur) system, found in *Escherichia coli* (Takahashi and Tokumoto 2002) and *Erwinia chrysanthemi* (Nachin et al. 2001). It is responsible for [Fe-S] cluster assembly under iron limitation or oxidative stress conditions (Nachin et al. 2001, Outten et al. 2004). The SUF gene cluster contains the genes *sufABCDSE*. SufS (also named CsdB) is a cysteine desulfurase (Mihara et al. 2000, 2002b). SufE is an activator of cysteine desulfurase, forming a complex with SufS and accepting sulfane sulfur from SufS (Loiseau et al. 2003, Outten et al. 2003, Ollagnier-de-Choudens et al. 2003a). SufA is a scaffold (Ollagnier-de-Choudens et al. 2003b). SufC is an ABC ATPase and forms a complex with SufB and SufD (Nachin et al. 2001, 2003, Rangachari et al. 2002, Outten et al. 2003). SufBCD contributes to the [Fe-S] cluster assembly under oxidative stress and iron limitation, but its exact role in the process is unknown.

More recently, a fourth bacterial [Fe-S] machinery, named CSD, was characterized in *Escherichia coli* (Loiseau et al. 2005). This is a simple gene cluster

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composed of *csdA-csdE* (formally *ygdK*). CsdA is a cysteine desulfurase (Mihara et al. 2000, Loiseau et al. 2005). CsdE is a SufE-like protein, activator of cysteine desulfurase. The CSD machinery in *Escherichia coli* constitutes a sulfur-generating system, which also contributes to [Fe-S] cluster biogenesis in vivo. The CSD system was proposed to supply [Fe-S] clusters for quinolinate synthetase NadA (Loiseau et al. 2005). The question remains what the scaffold molecule is, since at least one scaffold is required in all other [Fe-S] biosynthetic systems identified so far.

In summery, cysteine desulfurase activity (a NifS-like protein) is included in all above-described systems. It is considered the most important component of the [Fe-S] machinery. However, the function of NifS-like proteins is not limited to [Fe-S] cluster assembly. They also supply sulfur for the synthesis of a variety of biologically active molecules, such as thiamine, biotin, and molybdopterin. Moreover, NifS-like proteins also have selenocysteine lyase activity (for a review see Mihara and Esaki 2002a), providing the selenium for essential selenoproteins.

[Fe-S] proteins in chloroplasts

[Fe-S] proteins in plants have been reviewed by Imsande (1999), and Balk and Lobreaux (2005). As a major sub-cellular sink of iron for [Fe-S] proteins in plant cells, chloroplasts host various [Fe-S] proteins, involved in photosynthesis, nitrogen and sulfur assimilation, and a variety of other plastidic processes (Figure 1).

In the electron transfer chain of photosynthesis, electrons generated from H_2O molecules by light energy at photosystem II (PSII), are transferred to the cytochrome *b6/f* complex (b6f), containing a Rieske-type protein ([2Fe-2S]) (Breyton

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2000). The electrons are subsequently delivered to photosystem I (PSI). PSI contains an interpolypeptide [4Fe-4S] cluster, F(X), that bridges the PsaA and PsaB subunits, and two terminal [4Fe-4S] clusters, F(A) and F(B), that are bound to the PsaC subunit (Vassiliev et al. 2001). Through PSI, the electrons are passed to ferredoxin (Fd) which is a [2Fe-2S] protein. Finally, the electrons of ferredoxin can be used for the Calvin cycle of photosynthesis, or other reductive processes in chloroplasts. Ferredoxin-thioredoxin reductase (FTR), which is a [4Fe-4S] protein (Droux et al. 1987), is able to accept the electrons from ferredoxin and use them for light regulation of chloroplast enzymes.

The assimilation of sulfate into cysteine takes place mainly in chloroplasts (for a recent review see Pilon-Smits and Pilon 2005). Sulfate is activated by reaction with ATP to form adenosine-5-phosphosulfate (APS). APS reductase, which is a [4Fe-4S] protein, reduces APS to sulfite (Kopriva et al. 2001). Subsequently, sulfite reductase (SiR), containing a [4Fe-4S] cluster, reduces sulfite to sulfide (Krueger et al. 1982). Sulfide is incorporated into cysteine, which is the source of reduced sulfur in the cell.

In nitrogen assimilation, another central assimilatory pathway in chloroplasts, nitrite reductase (NiR), containing a [4Fe-4S] cluster, reduces nitrite to ammonia (Lancaster et al. 1979). Ammonia is subsequently coupled to glutamate, producing glutamine. Glutamate synthase also named GOGAT (Glutamine:2-Oxo-Glutarate Amido Transferase), a [3Fe-4S] protein, transfers a $-NH_2$ group from glutamine to α-keto-glutarate resulting in two glutamate molecules (Knaff et al. 1991). Glutamate and glutamine are the source of reduced nitrogen in the cell.

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Amidophosphoribosyltransferase (ATase), also named glutamine phosphoribosylpyrophosphate (PRPP) amidotransferase, is a [4Fe-4S] protein (Hung et al. 2004). It transfers a $-NH_2$ group from glutamine to PRPP resulting in 5-phosphoribosylamine, the first step in *de novo* biosynthesis of purine nucleotides.

Sirohydrochlorin ferrochelatase (SirB), which is a [2Fe-2S] protein, catalyzes the last step of siroheme biosynthesis utilizing iron (Raux-Deery et al. 2005). Interestingly, in plants, the siroheme is only used by sulfite reductase (SiR) and nitrite reductase (NiR), each of which contains a [4Fe-4S] cluster. In fact, plant SiR and NiR represent a unique family of siroheme-[4Fe-4S] enzymes, in which a [4Fe-4S] cluster and a siroheme are covalently bound, catalyzing a six-electron reduction (Crane and Getzoff 1996).

Pheophorbide a oxygenase (PaO, or LLS1), a Rieske-type [2Fe-2S] protein, is an enzyme involved in chlorophyll breakdown (Pruzinska et al. 2003). The PaO is located at the inner envelope of chloroplasts (Matile and Schellenberg 1996). Chlorophyll a oxygenase (CAO), a Rieske-type [2Fe-2S] protein, is involved in chlorophyll b formation from chlorophyll a (Tanaka et al. 1998). The CAO is likely located in the chloroplast stroma (Espineda et al. 1999). Tic55, a Rieske-type [2Fe-2S] protein, is a member of the chloroplastic inner envelope protein translocon (Caliebe et al. 1997). Choline monooxygenase (CMO) is a Rieske-type [2Fe-2S] protein, catalyzing the first step of synthesis of the osmoprotectant glycine betaine (Rathinasabapathi et al. 1997). Finally, dihydroxy acid dehydratase (DAD), the third enzyme in the branched-chain amino acid biosynthetic pathway, also contains a [2Fe-2S] cluster (Flint and Emptage 1988).

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[Fe-S] cluster biogenesis machinery in chloroplasts

It was found almost two decades ago that in vitro synthesized ferredoxin acquires its [2Fe-2S] cofactor after it is imported into chloroplasts (Takahashi et al. 1986; Li et al. 1990), suggesting that [Fe-S] clusters can be synthesized within this compartment independent of cytosol or other sub-cellular organelles, and thus that a separate [Fe-S] biogenesis machinery is present in chloroplasts. Biogenesis of [Fe-S] proteins in plants was recently reviewed by Balk and Lobreaux (2005). The chloroplast [Fe-S] biosynthetic machinery has been investigated using *Arabidopsis thaliana* as a model (Figure 2).

CpNifS (At1g08490) is a cysteine desulfurase (Leon et al. 2002; Pilon-Smits et al. 2002), converting cysteine into alanine and elemental sulfur, and providing sulfur for [Fe-S] assembly. Like all other NifS-like proteins, CpNifS has also selenocysteine lyase activity. Its selenocysteine lyase activity is 300-fold higher than its cysteine desulfurase activity. CpNifS is most similar to SufS among all three NifS-like proteins in *Escherichia coli*. So, CpNifS could also be termed CpSufS. As shown recently, CpNifS is essential for the [Fe-S] cluster formation activity of chloroplast stroma, and appears to be present in a ~600 kDa complex (Ye et al. 2005). In Arabidopsis, a second cysteine desulfurase is likely present in mitochondria (Kushnir et al. 2001), where another ISC-like [Fe-S] biogenesis machinery is thought to be active. Additionally, a third cysteine desulfurase activity is found in the cytosol (Heidenreich et al. 2005). It is ascribed to a NifS-like domain of the ABA3 protein,

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unlikely to be relevant for [Fe-S] synthesis, but functioning in the mobilization of sulfur for the molybdenum cofactor.

CpSufE (At4g26500) is a cysteine desulfurase activator (Chapter 3). It binds to and forms a heterotetrameric complex with CpNifS, stimulating cysteine desulfurase activity 40-fold and increasing substrate affinity of CpNifS toward cysteine. In vitro reconstitution assays showed that the capacity of CpNifS to assemble [2Fe-2S] clusters in ferredoxin was improved 20-fold by CpSufE. An essential cysteine residue (Cys65) in the CpSufE polypeptide is likely the acceptor site for intermediate sulfur. This cysteine residue is not required for binding to CpNifS. As a result, excess of a cysteine65-mutated CpSufE protein displayed a dominant-negative effect on the stimulatory activity of the wild-type protein. CpSufE also appeared to be present in a ~600 kDa chloroplast stromal complex. CpSufE protein is composed of a SufE domain and a BolA domain. The BolA protein was hypothesized to be a reductase interacting with a glutaredoxin and thus involved in oxidative stress response (Huynen et al. 2005). The role of the BolA domain in CpSufE protein remains to be investigated.

CpIscA (At1g10500) is a scaffold protein (Abdel-Ghany et al. 2005b). In vitro, by acquiring sulfur from CpNifS and ferrous iron from the media, CpIscA is able to assemble a [2Fe-2S] cluster within its dimeric polypeptides, resulting in holo-CpIscA. The holo-CpIscA, stably holding the transient [Fe-S] cluster, can be isolated and is able to deliver the [Fe-S] cluster to ferredoxin. Similarly, the presence of CpIscA scaffold improves the CpNifS-mediated [2Fe-2S] reconstitution in ferredoxin by ~2-fold. Like part of CpNifS and CpSufE, CpIscA is mostly present in a ~600 kDa

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chloroplast stromal complex. The likely role of the CpIscA in vivo is assembling [Fe-S] clusters for a subset of proteins, which remains to be determined.

Nfu1-3 are additional scaffold proteins (Leon et al. 2003; Touraine et al. 2004; Yabe et al. 2004). Nfu1 (At4g01940) and Nfu2 (At5g49940) were able to restore the growth of a scaffold-mutated yeast, the *Δisu1Δnfu1* strain, suggesting a role of them as a scaffold. However, the complementation by Nfu3 (At4g25910) was not evident, likely due to its low expression in yeast (Leon et al. 2003). Recombinant Nfu2 contained a labile [2Fe-2S] cluster, and could transfer it to apo-ferredoxin resulting in holo-ferredoxin (Leon et al. 2003; Yabe et al. 2004). In vivo mutation experiments further revealed that Nfu2 is required for assembling [4Fe-4S] clusters of photosystem I and [2Fe-2S] clusters of ferredoxin in chloroplasts (Touraine et al. 2004; Yabe et al. 2004). A subset but not all of chloroplast [Fe-S] proteins are affected in the *Nfu2* mutant, suggesting alternative scaffolds are functional in chloroplasts. Besides Nfu1-3, two more Nfu-like proteins have been reported in plants, Nfu4 and Nfu5, which are likely mitochondrial (Leon et al. 2003).

Arabidopsis SufB (At4g04770) is an ATPase (Xu et al. 2005), which can complement SufB deficiency in *Escherichia coli* during oxidative stress. SufC (At3g10670) is an ABC/ATPase (Xu and Moller 2004), which can partially rescue growth defects in an *Escherichia coli* SufC mutant during oxidative stress. SufD (At1g32500) is a protein with homology to the bacterial SufD, the mutation of which results in impaired embryogenesis and abnormal growth of Arabidopsis (Hjorth et al. 2005). Like their bacterial homologues, the chloroplast SufBCD form a complex (Xu and Moller 2004; Xu et al. 2005), displaying ABC/ATPase activity and likely

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involved in [Fe-S] cluster biogenesis. However, the exact role of the SufBCD complex in the process remains to be characterized. HCF101 (High Chlorophyll Fluorescence) (At3g24430) is a P-loop ATPase (Lezhneva et al. 2004), which was originally identified in a photosynthetic mutant screen (Stockel and Oelmuller 2004). It is required for the biogenesis of [4Fe-4S] clusters for photosystem I (PSI) and ferredoxin-thioredoxin reductase (FTR) in chloroplasts. However, a precise role of HCF101 in the process remains to be elucidated. APO1 (Accumulation of Photosystem One1) (At1g64810) is a member of an unknown gene family only found in vascular plants, which was originally identified in a photosystem I mutant screen (Amann et al. 2004). It is involved in the assembly of [4Fe-4S] cluster-containing complexes of chloroplasts, e.g. PSI and FTR. However, a direct connection between APO1 and [Fe-S] assembly needs to be shown.

In summary, chloroplasts likely have conserved a complete SUF-type machinery, including all components of SufABCDSE, for [Fe-S] biogenesis. It is reasonable that a SUF system, responsible for [Fe-S] biosynthesis under oxidative stress in bacteria, works well in the oxidative environment in chloroplasts. However, the entire chloroplast [Fe-S] machinery is apparently more complex, involving three more NifU-like proteins, a HCF101 that has no homologue in bacterial [Fe-S] machineries, and likely an APO1 that is unique in vascular plants. The importance and complexity of the chloroplast [Fe-S] machinery is illustrated by the phenotypes caused by mutation of individual machinery components, e.g. lethality for CpNifS and embryonic lethality for CpSufE (Abdel-Ghany et al. unpublished), dwarf and yellowish plants for Nfu2 (Touraine et al. 2004; Yabe et al. 2004), abnormal plastid

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structure and impaired embryogenesis for SufC or SufD (Xu and Moller 2004; Hjorth et al. 2005), and seedling lethality and high chlorophyll fluorescence for HCF101 (Lezhneva et al. 2004).

Implications of the [Fe-S] machinery for the homeostasis of iron and sulfur (and perhaps selenium) in plastids

By supplying [Fe-S] clusters to a variety of proteins, the plastid [Fe-S] biogenesis machinery is essential for many vital processes including photosynthesis, nitrogen assimilation, and sulfur assimilation (Figure 1). The efficiency of the [Fe-S] biosynthetic machinery directly affects those processes. Because of the importance of iron and sulfur in plant productivity and nutritional value, the impact of the plastid [Fe-S] machinery on the homeostasis of iron and sulfur is of particular interest.

Iron is an essential micronutrient to plants. Because iron is mainly present in soil as insoluble iron-oxide with low bioavailability, iron is one of the most limiting nutrients to plants. The uptake and transport of iron to leaf cells was reviewed by Curie and Briat (2003). In leaf cells, more than 90% of iron is located in chloroplasts (Terry and Abadia 1986). The molecular mechanism of iron transport into chloroplasts is not yet identified, but the Fe^{2+} transport across the chloroplast inner envelope membrane is measurable, and determined to be light-dependent. Fe^{2+} transport across the inner envelope membrane is stimulated by an electrochemical proton gradient, and reduced by negating the potential gradient, suggesting iron transport into chloroplasts is likely an Fe^{2+}/H^{+} symport transport mechanism (Shingles et al. 2002). Within the chloroplast, ~20% of iron is associated with thylakoid membranes, and the remaining

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~80% of iron is in the stroma (Bughio et al. 1997). However, a recent study suggested that ~40% of chloroplast iron is in the thylakoid (Abdel-Ghany et al. 2005a). In thylakoid membranes, iron is predominantly used for electron transfer. Approximately half of thylakoid iron is in photosystem I in the form of [4Fe-4S] clusters, and the remaining half is present in cytochromes in the form of hemes and Rieske-type [2Fe-2S] clusters. In the stroma, iron is used for assembling [Fe-S] clusters, hemes, Fe-SOD, etc. The excess of stromal iron is stored in ferritin (Petit et al. 2001). Because iron is one of the most limiting micronutrients to plants, plants might employ a sophisticated homeostasis mechanism to control cellular iron, particularly in plastids. At this point, the processes controlling plastid iron homeostasis are largely unknown. Our preliminary results show that CpNifS-CpSufE proteins, forming the cysteine desulfurase complex of plastid [Fe-S] biogenesis, are co-upregulated in the presence of excess iron and co-downregulated under limited iron (Chapter 5), suggesting the [Fe-S] biogenesis machinery is regulated by the plastid iron level. In bacteria, the ISC-type biogenesis machinery has an IscR. It is a transcription regulator controlling the expression of the ISC machinery by sensing the cellular iron level (Schwartz et al. 2001). Plants may have a similar sensing mechanism which remains to be investigated.

Conversely, the [Fe-S] biogenesis machinery may affect the available iron pool in a specific compartment. For instance, in yeast and mammalian cells, impaired mitochondrial [Fe-S] biogenesis results in iron overload in mitochondria (for a review see: Rouault and Tong 2005). Similar results were found for Arabidopsis mitochondria (Kushnir et al. 2001). Under particular physiological or pathological conditions when

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the activity of the plastid [Fe-S] machinery is abnormally changed, e.g. upregulated by selenate treatment or oxidative stress, or downregulated in continuous darkness (Chapter 5), it might affect iron homeostasis. Investigations on how the [Fe-S] machinery is regulated by iron levels, and how a modulated machinery affects iron homeostasis, represents a promising research area. Transgenic plants in which the expression levels of CpNifS and/or CpSufE (WT or mutant) are modulated from high overexpression to knockout, will be a useful tool in such studies. A collection of such transgenics is currently being made in our lab.

Sulfur is a macronutrient, widely present in a variety of biological molecules. Generally, it is not limiting for plants. Sulfur metabolism in plants has been recently reviewed by Pilon-Smits and Pilon (2005). The plastid is the predominant compartment for sulfur assimilation from sulfate to cysteine. In the process, out of four enzymes involved, two are [Fe-S] proteins: APS reductase and sulfite reductase (SiR) (Figure 1). Thus, sulfur assimilation is likely dependent on the plastid [Fe-S] machinery. In particular, the step catalyzed by SiR is expected to be dependent on the [Fe-S] machinery. The catalytic enzyme SiR employs a unique siroheme-[4Fe-4S] as its prosthetic group, requiring not only a direct incorporation of a [4Fe-4S] cluster but also a sirohydrochlorin ferrochelatase (SirB), which is a [2Fe-2S] protein, for synthesizing the siroheme. Moreover, the six electron reduction catalyzed by SiR needs ferredoxin, a [2Fe-2S] protein, for providing electrons. In the final step of sulfur assimilation, sulfide is incorporated into cysteine, which is the source of reduced sulfur in plastids. To supply reduced sulfur from cysteine, the CpNifS-CpSufE activity is required, which provides sulfur not only for [Fe-S] biogenesis, but also likely for

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synthesis of various sulfur-containing biomolecules (Mihara and Esaki 2002). It is likely that the [Fe-S] machinery is important to the sulfur assimilation via CpNifS-CpSufE. On the other hand, the cellular sulfur level likely affects the activity of the [Fe-S] machinery. Our preliminary results show that the scaffold protein CpIscA is downregulated under sulfur starvation (Chapter 5). The molecular mechanism of such regulation remains to be investigated.

Selenium is a group VIa metalloid without evident essentiality for plants. It is metabolized by plants in the same pathway as sulfur, because of their chemical similarity. Selenium metabolism in higher plants has been reviewed by Terry and coworkers (2000). Much of selenium metabolism takes place in chloroplasts. Selenium is assimilated into seleno-cysteine and seleno-methionine, which can be nonspecifically incorporated into proteins by replacing sulfur amino acids. Since cysteine residues are important for structure and function of proteins, the substitution of cysteine by seleno-cysteine results in loss of protein function (Brown and Shrift 1982). Plants can employ the seleno-cysteine lyase (SL) activity of NifS-like proteins to break seleno-cysteine into alanine and selenide, thereby avoiding selenium toxicity. In an initial approach, Arabidopsis was shown to acquire enhanced selenium tolerance and accumulation when overexpressing a mouse SL (Pilon et al. 2003). Arabidopsis has its own seleno-cysteine lyase, the CpNifS in chloroplasts. This is also the cysteine desulfurase component of the plastid [Fe-S] biogenesis machinery. Recently, transgenic Arabidopsis was obtained overexpressing the CpNifS in plastids. It too displayed higher tolerance and accumulation of selenium (Van Hoewyk et al. 2005). Therefore, altering levels of this [Fe-S] machinery component clearly affects selenium

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metabolism. On the other hand, the cellular selenium level affects the activity of the [Fe-S] machinery. Selenate treatment upregulated expression of CpNifS and CpSufE (Chapter 5). Understanding how the plastid [Fe-S] biogenesis machinery, particularly the CpNifS-CpSufE complex, is implicated in selenium homeostasis, will be applicable in selenium phytoremediation, using plants to cleanup areas contaminated with selenium.

Scope of this thesis

The goal of this work was to gain insight into the mechanisms of [Fe-S] cluster assembly in chloroplasts. To this aim we studied [Fe-S] cluster biogenesis in chloroplasts *in vitro*. The biochemical role of three components, CpNifS, CpSufE and CpIscA, was investigated. The cysteine desulfurase (CysD) CpNifS was found to be required for [Fe-S] formation in chloroplast stroma. CpSufE and CpIscA are newly identified components, and were found to be a CysD activator and a molecular scaffold, respectively. In addition to these biochemical/*in vitro* studies, a start was made with the investigation of roles of these gene products *in vivo*, including expression analysis and transgenic/knockout approaches.

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References

Abdel-Ghany, S.E., Muller-Moule, P., Niyogi, K.K., Pilon, M., and Shikanai, T. (2005a) Two P-type ATPases are required for copper delivery in Arabidopsis thaliana chloroplasts. *Plant Cell* 17, 1233-1251

Abdel-Ghany, S.E., Ye, H., Garifullina, G.F., Zhang, L., Pilon-Smits, E.A.H., and Pilon, M. (2005b) Iron-sulfur cluster biogenesis in chloroplasts. Involvement of the scaffold protein CpIscA. *Plant Physiol.* 138, 161-172

Agar, J.N., Yuvaniyama, P., Jack, R.F., Cash, V.L., Smith, A.D., Dean, D.R., and Johnson, M.K. (2000a) Modular organization and identification of a mononuclear iron-binding site within the NifU protein. *J. Biol. Inorg. Chem.* 5, 167-177

Agar, J.N., Krebs, C., Frazzon, J., Huynh, B.H., Dean, D.R., and Johnson, M.K. (2000b) IscU as a scaffold for iron-sulfur cluster biosynthesis: sequential assembly of [2Fe-2S] and [4Fe-4S] clusters in IscU. *Biochemistry* 39, 7856-7862

Amann, K., Lezhneva, L., Wanner, G., Herrmann, R.G., and Meurer, J. (2004) Accumulation of photosystem one1, a member of a novel gene family, is required for accumulation of [4Fe-4S] cluster-containing chloroplast complexes and antenna proteins. *Plant Cell* 16, 3084-3097

Balk, J., and Lobreaux, S. (2005) Biogenesis of iron-sulfur proteins in plants. *Trends Plant Sci.* 10, 324-331

Beinert, H., Holm, R.H., and Munck, E. (1997) Iron-Sulfur Clusters: Nature's Modular Multipurpose Structures. *Science* 277, 653-659

Beinert, H. and Kiley, P.J. (1999) [Fe-S] proteins in sensing and regulatory functions. *Curr. Opin. Chem. Biol.* 3, 152-157

Beinert, H. (2000) Iron-sulfur proteins: ancient structures, still full of surprises. *J. Biol. Inorg. Chem.* 5, 2-15

Breyton, C. (2000) The cytochrome b(6)f complex: structural studies and comparison with the bc(1) complex. *Biochim. Biophys. Acta* 1459, 467-474

Brown, T.A., and Shrift, A. (1982) Selenium: toxicity and tolerance in higher plants. *Biol. Rev.* 57, 59-84

Bughio, N., Takahashi, M., Yoshimura, E., Nishizawa, N.K., and Mori, S. (1997) Light-dependant iron transport into isolated barley chloroplasts. *Plant Cell Physiol.* 38, 101-105

Caliebe, A., Grimm, R., Kaiser, G., Lubeck, J., Soll, J., and Heins, L. (1997) The Chloroplastic protein import machinery contains a Rieske-type iron-sulfur cluster and a mononuclear iron-binding protein. *EMBO J.* 16, 7342-7350

Crane, B. R., and Getzoff, E. D. (1996) The relationship between structure and function for the sulfide reductases. *Curr. Opin. Struct. Biol.* 6, 744–756

Curie, C., and Briat, J.F. (2003) iron transport and signaling in plants. *Annu. Rev. Plant Biol.* 54, 183-206

Ding, H., and Clark, R.J. (2004a) Characterization of iron binding in IscA, an ancient iron-sulphur cluster assembly protein. *Biochem. J.* 15, 433-440

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Ding, H., Clark, R.J., and Ding, B. (2004b) IscA mediates iron delivery for assembly of iron-sulfur clusters in IscU under the limited accessible free iron conditions. *J. Biol. Chem.* 279, 37499-37504

Ding, B., Smith, E.S., and Ding, H. (2005) Mobilization of the iron centre in IscA for the iron-sulphur cluster assembly in IscU. *Biochem. J.* 389, 797-802

Droux, M., Jacquot, J.P., Miginac-Maslow, M., Gadal, P., Huet, J.C., Crawford, N.A., Yee, B.C., and Buchanan, B.B. (1987) Ferredoxin-thioredoxin reductase, an iron-sulfur enzyme linking light to enzyme regulation in oxygenic photosynthesis: purification and properties of the enzyme from C3, C4, and cyanobacterial species. *Arch. Biochem. Biophys.* 252, 426-439

Espineda, C.E., Linford, A.S., Devine, D., and Brusslan, J.A. (1999) The AtCAO gene, encoding chlorophyll a oxygenase, is required for chlorophyll b synthesis in Arabidopsis thaliana. *Proc. Natl. Acad. Sci. USA.* 96, 10507-10511

Evans, D.J., Jones, R., Woodley, P.R., Wilborn, J.R., and Robson, R.L. (1991) Nucleotide sequence and genetic analysis of the Azotobacter chroococcum nifUSVWZM gene cluster, including a new gene (nifP) which encodes a serine acetyltransferase. *J. Bacteriol.* 173, 5457-5469

Flint, D.H., and Emptage, M.H. (1988) Dihydroxy acid dehydratase from spinach contains a [2Fe-2S] cluster. *J. Biol. Chem.* 263, 3558-3564

Fu, W., Jack, R.F., Morgan, T.V., Dean, D.R., and Johnson, M.K. (1994) nifU gene product from Azotobacter vinelandii is a homodimer that contains two identical [2Fe-2S] clusters. *Biochemistry* 33, 13455-13463

Heidenreich, T., Wollers, S., Mendel, R.R., and Bittner, F. (2005) Characterization of the NifS-like domain of ABA3 from Arabidopsis thaliana provides insight into the mechanism of molybdenum cofactor sulfuration. *J. Biol. Chem.* 280, 4213-4218

Hjorth, E., Hadfi, K., Zauner, S., and Maier, U.G. (2005) Unique genetic compartmentalization of the SUF system in cryptophytes and characterization of a SufD mutant in Arabidopsis thaliana. *FEBS Lett.* 579, 1129-1135

Hoff, K.G., Silberg, J.J., and Vickery, L.E. (2000) Interaction of the iron-sulfur cluster assembly protein IscU with the Hsc66/Hsc20 molecular chaperone system of Escherichia coli. *Proc. Natl. Acad. Sci. USA.* 97, 7790-7795

Hung, W.F., Chen, L.J., Boldt, R., Sun, C.W., Li, H.M. (2004) Characterization of Arabidopsis glutamine phosphoribosyl pyrophosphate amidotransferase-deficient mutants. *Plant Physiol.* 135, 1314-1323

Huynen MA, Spronk CA, Gabaldon T, and Snel B. (2005) Combining data from genomes, Y2H and 3D structure indicates that BolA is a reductase interacting with a glutaredoxin. *FEBS Lett.* 579, 591-596

Imsande, J. (1999) Iron-sulfur clusters: Formation, perturbation, and physiological functions. *Plant Physiol. Biochem.* 37, 87-97

Jacobson, M.R., Brigle, K.E., Bennett, L.T., Setterquist, R.A., Wilson, M.S., Cash, V.L., Beynon, J., Newton, W.E., and Dean, D.R. (1989) Physical and genetic map of the major nif gene cluster from Azotobacter vinelandii. *J. Bacteriol.* 171, 1017-1027

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Johnson, D.C., Dos Santos, P.C., and Dean, D.R. (2005) NifU and NifS are required for the maturation of nitrogenase and cannot replace the function of isc-gene products in Azotobacter vinelandii. *Biochem. Soc. Trans.* 33, 90-93

Johnson, D., Dean, D.R., Smith, A.D., and Johnson, M.K. (2005) Structure, function, and formation of biological iron-sulfur clusters. *Annu. Rev. Biochem.* 74, 247–281

Kato, S., Mihara, H., Kurihara, T., Takahashi, Y., Tokumoto, U., Yoshimura, T., Esaki, N. (2002) Cys-328 of IscS and Cys-63 of IscU are the sites of disulfide bridge formation in a covalently bound IscS/IscU complex: implications for the mechanism of iron-sulfur cluster assembly. *Proc. Natl. Acad. Sci. USA.* 99, 5948-5952

Knaff, D.B., Hirasawa, M., Ameyibor, E., Fu, W., and Johnson, M.K. (1991) Spectroscopic evidence for a [3Fe-4S] cluster in spinach glutamate synthase. *J. Biol. Chem.* 266, 15080- 15084

Kopriva, S., Buchert, T., Fritz, G., Suter, M., Weber, M., Benda, R., Schaller, J., and Feller, U., Schurmann, P., Schunemann, V., Trautwein, A.X., Kroneck, P.M., and Brunold, C. (2001) Plant adenosine 5'-phosphosulfate reductase is a novel iron-sulfur protein. *J. Biol. Chem.* 276, 42881-42886

Krebs, C., Agar, J.N., Smith, A.D., Frazzon, J., Dean, D.R., Huynh, B.H., and Johnson, M.K. (2001) IscA, an alternate scaffold for [Fe-S] cluster biosynthesis. *Biochemistry* 40, 14069-14080

Krueger, R.J., and Siegel, L.M. (1982) Spinach siroheme enzymes: Isolation and characterization of ferredoxin-sulfite reductase and comparison of properties with ferredoxin-nitrite reductase. *Biochemistry* 21, 2892-2904

Kushnir, S., Babiychuk, E., Storozhenko, S., Davey, M.W., Papenbrock, J., De Rycke, R., Engler, G., Stephan, U.W., Lange, H., Kispal, G., Lill, R., and Van Montagu, M. (2001) A mutation of the mitochondrial ABC transporter Sta1 leads to dwarfism and chlorosis in the Arabidopsis mutant starik. *Plant Cell* 13, 89-100

Lancaster, J.R., Vega, J.M., Kamin, H., Orme-Johnson, N.R., Orme-Johnson, W.H., Krueger, R.J., Siegel, L.M. (1979) Identification of the iron-sulfur center of spinach ferredoxin-nitrite reductase as a tetranuclear center, and preliminary EPR studies of mechanism. *J. Biol. Chem.* 254, 1268-1272

Leon, S., Touraine, B., Briat, J.F., and Lobreaux, S. (2002) The AtNFS2 gene from Arabidopsis thaliana encodes a NifS-like plastidial cysteine desulphurase. *Biochem. J.* 366, 557-564

Leon, S., Touraine, B., Ribot, C., Briat, J.F., and Lobreaux, S. (2003) Iron-sulphur cluster assembly in plants: distinct NFU proteins in mitochondria and plastids from Arabidopsis thaliana. *Biochem. J.* 371, 823-830

Lezhneva, L., Amann, K., and Meurer, J. (2004) The universally conserved HCF101 protein is involved in assembly of [4Fe-4S]-cluster-containing complexes in Arabidopsis thaliana chloroplasts. *Plant J.* 37, 174-185

Li, H.M., Theg, S.M., Bauerle, C.M., and Keegstra, K. (1990) Metal-ion-center assembly of ferredoxin and plastocyanin in isolated chloroplasts. *Proc. Natl. Acad. Sci. USA.* 87, 6748-6752

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Lill, R. and Muhlenhoff, U. (2005) Iron-sulfur-protein biogenesis in eukaryotes. *Trends Biochem. Sci.* 30, 133-141

Loiseau, L., Ollagnier-de-Choudens, S., Nachin, L., Fontecave, M., and Barras F. (2003) Biogenesis of [Fe-S] cluster by the bacterial Suf system: SufS and SufE form a new type of cysteine desulfurase. *J. Biol. Chem.* 278, 38352-38359

Loiseau, L., Ollagnier-de Choudens, S., Lascoux, D., Forest, E., Fontecave, M., and Barras, F. (2005) Analysis of the heteromeric CsdA-CsdE cysteine desulfurase, assisting [Fe-S] cluster biogenesis in Escherichia coli. *J. Biol. Chem.* 280, 26760-26769

Mansy, S.S., Wu, G., Surerus, K.K., and Cowan, J.A. (2002) Iron-sulfur cluster biosynthesis. Thermatoga maritima IscU is a structured iron-sulfur cluster assembly protein. *J. Biol. Chem.* 277, 21397-21404

Matile, P., and Schellenberg, M. (1996) The cleavage of phaeophorbide a is located in the envelope of barley gerontoplasts. *Plant Physiol. Biochem.* 34, 55-59

Mihara, H., Kurihara, T., Yoshimura, T., and Esaki, N. (2000) Kinetic and mutational studies of three NifS homologs from Escherichia coli: mechanistic difference between L-cysteine desulfurase and L-selenocysteine lyase reactions. *J. Biochem.* 127, 559-567

Mihara, H., and Esaki, N. (2002a) Bacterial cysteine desulfurases: their function and mechanisms. *Appl. Microbiol. Biotechnol.* 60, 12-23

Mihara, H., Fujii, T., Kato, K., Hata, Y., and Esaki, N. (2002b) Structure of external aldimine of Escherichia coli CsdB, an IscS/NifS homolog: implications for its specificity toward selenocysteine. *J. Biochem.* 131, 679–685

Nachin, L., El Hassouni, M., Loiseau, L., Expert, D., and Barras, F. (2001) SoxR-dependant response to oxidative stress and virulence of Erwinia chrysanthemi: the key role of SufC, an orphan ABC ATPase. *Mol. Microbiol.* 39, 960-972

Nachin, L., Loiseau, L., Expert, D., and Barras, F. (2003) SufC: an unorthodox cytoplasmic ABC/ATPase required for [[Fe-S]] biogenesis under oxidative stress. *EMBO J.* 22, 427-437

Ollagnier-de-Choudens,. S., Mattioli, T., Takahashi, Y., and Fontecave, M. (2001) Iron-sulfur cluste assembly: characterization of IscA and evidence for a specific and functional complex with ferredoxin. *J. Biol. Chem.* 276, 22604-22607

Ollagnier-de-Choudens, S., Lascoux, D., Loiseau, L., Barras, F., Forest, E., and Fontecave, M. (2003a) Mechanistic studies of the SufS-SufE cysteine desulfurase: evidence for sulfur transfer from SufS to SufE. *FEBS Lett.* 555, 263-267

Ollagnier-de-Choudens, S., Nachin, L., Sanakis, Y., Loiseau, L., Barras, F., and Fontecave, M. (2003b) SufA from Erwinia chrysanthemi. Characterization of a scaffold protein required for iron-sulfur cluster assembly. *J. Biol. Chem.* 278, 17993-18001

Ollagnier-de-Choudens, S., Sanakis, Y., and Fontecave, M. (2004) SufA/IscA: reactivity studies of a class of scaffold proteins involved in [Fe-S] cluster assembly. *J. Biol. Inorg. Chem.* 9, 828-838

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Outten, F.W., Wood, M.J., Munoz, F.M., and Storz, G. (2003) The SufE protein and the SufBCD complex enhance SufS cysteine desulfurase activity as part of a sulfur transfer pathway for [Fe-S] cluster assembly in Escherichia coli. *J. Biol. Chem.* 278, 45713-45719

Outten, F.W., Djaman, O., and Storz, G. (2004) A suf operon requirement for [Fe-S] cluster assembly during iron starvation in Escherichia coli. *Mol. Microbiol.* 52, 861-872

Petit, J.M., Briat, J.F., and Lobreaux, S. (2001) Structure and differential expression of the four members of the Arabidopsis thaliana ferritin gene family. *Biochem. J.* 359, 575-582

Pilon, M., Owen, J.D., Garifullina, G.F., Kurihara, T., Mihara, H., Esaki, N., and Pilon-Smits, E.A. (2003) Enhanced selenium tolerance and accumulation in transgenic Arabidopsis expressing a mouse selenocysteine lyase. *Plant Physiol.* 131, 1250-1257

Pilon, M., Abdel-Ghany, S., Ye, H., Van Hoewyk, D., and Pilon-Smits, E.A.H. (2005) Biogenesis of iron-sulfur cluster proteins in plastids. In: *Genetic engineering principles and methods*, Wiley, New York

Pilon-Smits, E.A.H., Garifullina, G.F., Abdel-Ghany, S., Kato, S., Mihara, H., Hale, K.L., Burkhead, J.L., Esaki, N., Kurihara, T., and Pilon, M. (2002) Characterization of a NifS-like chloroplast protein from Arabidopsis. Implications for its role in sulfur and selenium metabolism. *Plant Physiol.* 130, 1309-1318

Pilon-Smits, E.A.H., and Pilon, M. (2005) Sulfur metabolism in plastids. In: Wise RR, Hoober JK. (Eds) "*Advances in Photosynthesis and Respiration - The Structure and Function of Plastids*". Kluwer Academic Publishers, Dordrecht, The Netherlands. In press

Pruzinska, A., Tanner, G., Anders, I., Roca, M., and Hortensteiner, S. (2003) Chlorophyll breakdown: pheophorbide a oxygenase is a Rieske-type iron-sulfur protein, encoded by the accelerated cell death 1 gene. *Proc. Natl. Acad. Sci. USA.*.100, 15259-15264

Rangachari, K., Davis, C.T., Eccleston, J.F., Hirst, E.M., Saldanha, J.W., Strath, M., and Wilson, R.J. (2002) SufC hydrolyzes ATP and interacts with SufB from Thermotoga maritima. *FEBS Lett.* 514, 225-228

Rathinasabapathi, B., Burnet, M., Russell, B.L., Gage, D.A., Liao, P.C., Nye, G.J., Scott, P., Golbeck, J.H., and Hanson, A.D. (1997) Choline monooxygenase, an unusual iron-sulfur enzyme catalyzing the first step of glycine betaine synthesis in plants: prosthetic group characterization and cDNA cloning. *Proc. Natl. Acad. Sci. USA.* 94, 3454-3458

Raux-Deery, E., Leech, H.K., Nakrieko, K.A., McLean, K.J., Munro, A.W., Heathcote, P., Rigby, S.E., Smith, A.G., and Warren, M.J. (2005) Identification and characterization of the terminal enzyme of siroheme biosynthesis from Arabidopsis thaliana: a plastid-located sirohydrochlorin ferrochelatase containing a 2FE-2S center. *J. Biol. Chem.* 280, 4713-4721

Rouault, T.A. and Tong, W.H. (2005) Iron-sulphur cluster biogenesis and mitochondrial iron homeostasis. *Nat. Rev. Mol. Cell. Biol.* 6, 345-351

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Schwartz, C.J., Djaman, O., Imlay, J.A., and Kiley, P.J. (2000) The cysteine desulfurase, IscS, has a major role in in vivo [Fe-S] cluster formation in Escherichia coli. *Proc. Natl. Acad. Sci. USA.* 97, 9009-9014

Schwartz, C.J., Giel, J.L., Patschkowski, T., Luther, C., Ruzicka, F.J., Beinert, H., and Kiley, P.J. (2001) IscR, an [Fe-S] cluster-containing transcription factor, represses expression of *Escherichia coli* genes encoding [Fe-S] cluster assembly proteins. *Proc. Natl. Acad. Sci. USA.* 98, 14895-14900

Shingles, R., North, M., and McCarty, R.E. (2002) ferrous iron transport across chloroplast inner envelope membranes. *Plant Physiol.* 128, 1022-1030

Silberg, J.J., Hoff, K.G., Tapley, T.L., and Vickery, L.E. (2001) The Fe/S assembly protein IscU behaves as a substrate for the molecular chaperone Hsc66 from Escherichia coli. *J. Biol. Chem.* 276, 1696-1700

Smith, A.D., Agar, J.N., Johnson, K.A., Frazzon, J., Amster, I.J., Dean, D.R., and Johnson, M.K. (2001) Sulfur transfer from IscS to IscU: the first step in iron-sulfur cluster biosynthesis. *J. Am. Chem. Soc.* 123, 11103-11104

Stockel, J., and Oelmuller, R. (2004) A novel protein for photosystem I biogenesis. *J. Biol. Chem.* 279, 10243-10251

Takahashi, Y., Mitsui, A., Hase, T., and Matsubara, H. (1986) Formation of the Iron-Sulfur Cluster of Ferredoxin in Isolated Chloroplasts. *Proc. Natl. Acad. Sci. USA.* 83, 2434-2437

Takahashi, Y., and Nakamura, M. (1999) Functional assignment of the ORF2-iscS-iscU-iscA-hscB-hscA-fdx-ORF3 gene cluster involved in the assembly of [Fe-S] clusters in Escherichia coli. *J. Biochem.* 126, 917-926

Takahashi, Y., and Tokumoto, U. (2002) A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. *J. Biol. Chem.* 277, 28380-28383

Tanaka, A., Ito, H., Tanaka, R., Tanaka, N.K., Yoshida, K., and Okada, K. (1998) Chlorophyll an oxygenase (CAO) is involved in chlorophyll b formation from chlorophyll a. *Proc. Natl. Acad. Sci. USA.* 95, 12719-12723

Terry, N., and Abadia, J. (1986) Function of iron in chloroplasts. *J. Plant Nutr.* 9, 609-646

Terry, N., Zayed, A.M., de Souza, M.P., and Tarun, A.S. (2000) Selenium in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 51, 401-432

Touraine, B., Boutin, J.P., Marion-Poll, A., Briat, J.F., Peltier, G., and Lobreaux, S. (2004) Nfu2: a scaffold protein required for [4Fe-4S] and ferredoxin iron-sulphur cluster assembly in Arabidopsis chloroplasts. *Plant J.* 40, 101-111

Urbina, H.D., Silberg, J.J., Hoff, K.G., and Vickery, L.E. (2001) Transfer of sulfur from IscS to IscU during Fe/S cluster assembly. *J. Biol. Chem.* 276, 44521-44526

Van Hoewyk, D., Garifullina, G.F., Ackley, A.R., Abdel-Ghany, S.E., Marcus, M.A., Fakra, S., Ishiyama, K., Ioue, E., Pilon, M., Takahashi, H., and Pilon-Smits, E.A.H. (2005) Overexpression of AtCpNifS enhances selenium tolerance and accumulation in Arabidopsis. *Plant Physiol.* In press

Vassiliev, I.R., Antonkine, M.L., and Golbeck, J.H. (2001) Iron-sulfur clusters in type I reaction centers. *Biochim. Biophys. Acta* 1507, 139-160

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Xu, X.M., and Moller, S.G. (2004) AtNAP7 is a plastidic SufC-like ATP-binding cassette /ATPase essential for Arabidopsis embryogenesis. *Proc. Natl. Acad. Sci. USA.* 101, 9143-9148

Xu, X.M., Adams, S., Chua, N.H., and Moller, S.G. (2005) AtNAP1 represents an atypical SufB protein in Arabidopsis plastids. *J. Biol. Chem.* 280, 6648-6654

Yabe, T., Morimoto, K., Kikuchi, S., Nishio, K., Terashima, I., and Nakai, M. (2004) The Arabidopsis chloroplastic NifU-like protein CnfU, which can act as an iron-sulfur cluster scaffold protein, is required for biogenesis of ferredoxin and photosystem I. *Plant Cell* 16, 993-1007

Ye, H., Garifullina, G.F., Abdel-Ghany, S.E., Zhang, L., Pilon-Smits, E.A., and Pilon, M. (2005) The chloroplast NifS-like protein of Arabidopsis thaliana is required for iron-sulfur cluster formation in ferredoxin. *Planta* 220, 602-608

Yuvaniyama, P., Agar, J.N., Cash, V.L., Johnson, M.K., and Dean, D.R. (2000) NifS-directed assembly of a transient [2Fe-2S] cluster within the NifU protein. *Proc. Natl. Acad. Sci. USA.* 97, 599-604

Zheng, L., and Dean, D.R. (1994) Catalytic formation of a nitrogenase iron-sulfur cluster. *J. Biol. Chem.* 269, 18723-18726

Zheng, L., White, R.H., and Dean, D.R. (1997) Purification of the Azotobacter vinelandii nifV-encoded homocitrate synthase. *J. Bacteriol.* 179, 5963-5966

Zheng, L., Cash, V.L., Flint, D.H., and Dean, D.R. (1998) Assembly of iron-sulfur clusters. Identification of an iscSUA-hscBA-fdx gene cluster from Azotobacter vinelandii. *J. Biol. Chem.* 273, 13264-13272

Zheng, L., White, R.H., Cash, V.L., Jack, R.F., and Dean, D.R. (1993) Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. *Proc. Natl. Acad. Sci. USA.* 90, 2754-2758

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Figure Legends:

Figure 1. Plastidic [Fe-S] proteins, labeled with circles, are shown in pathways they are involved in. b6f: cytochrome *b6/f* complex, PSI: photosystem I, Fd: ferredoxin, FTR: ferredoxin-thioredoxin reductase, APS reductase: adenosine-5-phosphosulfate reductase, SiR: sulfite reductase, NiR: nitrite reductase, GOGAT: Glutamine:2-Oxo-Glutarate Amido Transferase, ATase: Amidophosphoribosyltransferase, PaO: Pheophorbide a oxygenase, CAO: Chlorophyll a oxygenase, Tic55: translocon at the inner envelope membrane of chloroplasts, CMO: Choline monooxygenase, DAD: dihydroxy acid dehydratase.

Figure 2. Plastidic [Fe-S] biogenesis machinery. In chloroplast stroma, CpNifS and CpSufE form a putative two-component cysteine desulfurase. It breaks down cysteine and provides elemental sulfur for [Fe-S] cluster synthesis. Iron is mobilized via an unknown mechanism. A transient [Fe-S] cluster is synthesized in a scaffold molecule either CpIscA or Nfu proteins, and subsequently delivered to target proteins for maturation. CpNifS, CpSufE and CpIscA are in part present in a 600 kDa plastidic complex. SufBCD and HCF101, which have ATPase activity, and APO1 proteins are thought to also be involved in plastidic [Fe-S] biogenesis, but their roles are unknown.

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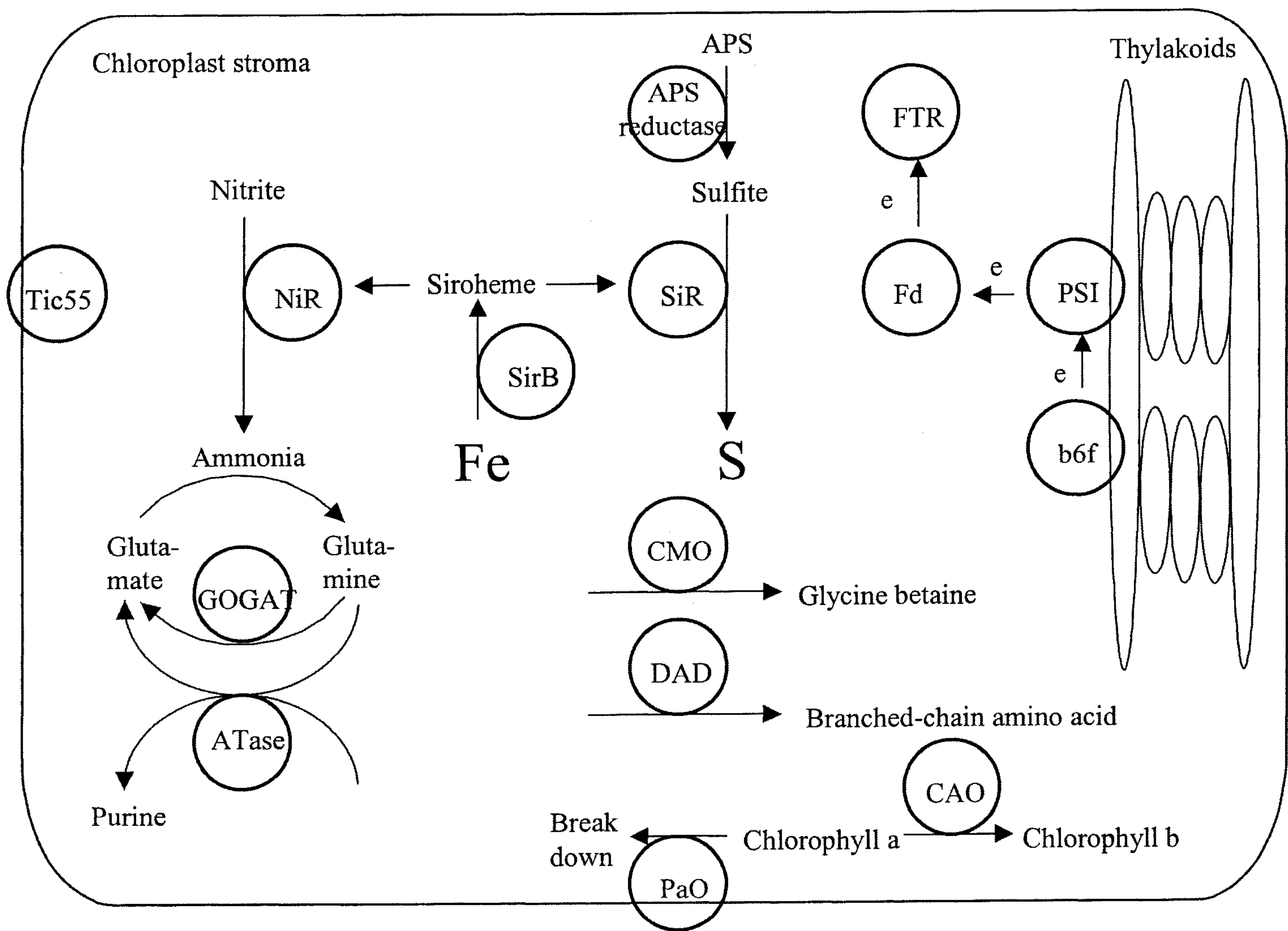

Figure 1. Plastidic [Fe-S] proteins.

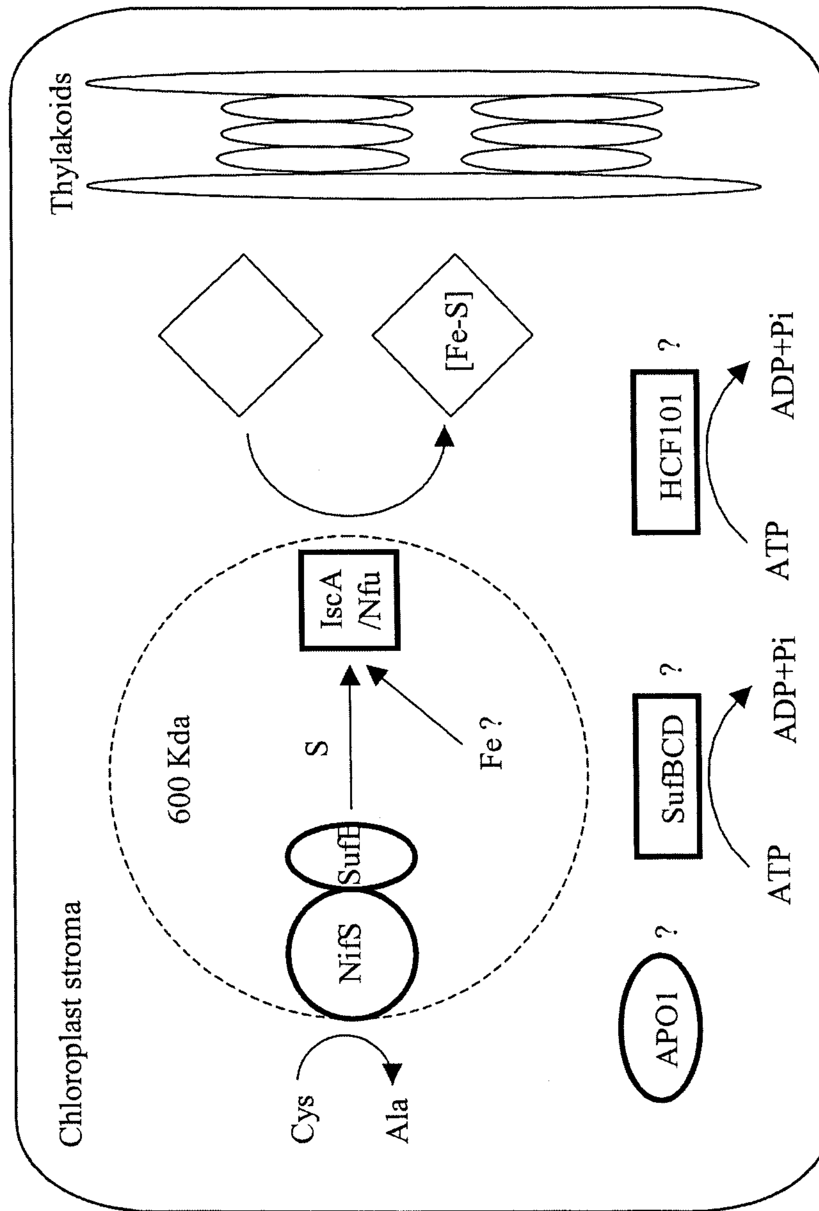

Figure 2. Plastid [Fe-S] biogenesis machinery.

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Chapter 2

In this Chapter the role of the cysteine desulfurase (CysD) CpNifS in [Fe-S] formation in ferredoxin was studied. It was found that the CpNifS is required for the [Fe-S] formation activity of chloroplast stroma. Therefore it is the first characterized player of the [Fe-S] biosynthesis machinery in chloroplasts. All experiments were carried out by Hong Ye. Coauthors helped by teaching critical methods and making figures.

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ORIGINAL ARTICLE

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The chloroplast NifS-like protein of *Arabidopsis thaliana* is required for iron–sulfur cluster formation in ferredoxin

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Abstract Plastids are known to be able to synthesize their own iron–sulfur clusters, but the biochemical machinery responsible for this process is not known. In this study it is investigated whether CpNifS, the chloroplastic NifS-like cysteine desulfurase of *Arabidopsis thaliana* (L.) Heynh. is responsible for the release of sulfur from cysteine for the biogenesis of iron–sulfur (Fe–S) clusters in chloroplasts. Using an in vitro reconstitution assay it was found that purified CpNifS was sufficient for Fe–S cluster formation in ferredoxin in the presence of cysteine and a ferrous iron salt. Antibody-depletion experiments using stromal extract showed that CpNifS is also essential for the Fe–S cluster formation activity of chloroplast stroma. The activity of CpNifS in the stroma was 50- to 80-fold higher than that of purified CpNifS on a per-protein basis, indicating that other stromal factors cooperate in Fe–S cluster formation. When stromal extract was separated on a gel-filtration column, most of the CpNifS eluted as a dimer of 86 kDa, but a minor fraction of the stromal CpNifS eluted at a molecular weight of approx. 600 kDa, suggesting the presence of a multi-protein complex. The possible nature of the interacting proteins is discussed.

Keywords *Arabidopsis* · Chloroplast · Ferredoxin · NifS-like protein · Iron–sulfur cluster

Abbreviations CpNifS: Chloroplast NifS-like protein · fd: Ferredoxin · PLP: Pyridoxal-5′-phosphate · DTT: Dithiothreitol · Fe–S: Iron–sulfur

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Introduction

In plant chloroplasts, iron–sulfur (Fe–S) clusters are required for the function of the cytochrome *b*/*f* complex (one 2Fe–2S cluster), photosystem I (three 4Fe–4S clusters) and ferredoxin (fd; one 2Fe–2S cluster) and thus are pivotal for photosynthetic electron transport (Raven et al. 1999). In eukaryotes, an Fe–S assembly machinery is present in the mitochondria, and evidence obtained in yeast and plants indicates that this machinery may even aid Fe–S cluster formation in the cytosol (Kispal et al. 1999; Kushnir et al. 2001). Iron–sulfur cluster assembly in ferredoxin was observed in isolated chloroplasts with cysteine as the sulfur donor, a reaction that further required light or ATP and NADPH (Takahashi et al. 1986, 1990). Newly imported ferredoxin, which is imported from the cytosol as a precursor in the apo-form without a cofactor (Pilon et al. 1992), was efficiently converted to the Fe–S-containing holoprotein (Li et al. 1990) even in the absence of cytosol (Pilon et al. 1995). Thus, chloroplasts may have their own Fe–S synthesis machinery.

NifS-like proteins are pyridoxal-5′-phosphate (PLP)-dependent enzymes with both cysteine desulfurase and selenocysteine lyase activities that can be placed into two groups based on sequence similarity (Mihara et al. 1997). In bacteria and in yeast mitochondria, cysteine desulfurases of group I with structural similarity to the NifS enzyme from *Azotobacter vinelandii* (Zheng et al. 1993) provide sulfur for Fe–S formation (for reviews, see Lill and Kispal 2000; Frazzon et al. 2002). Bacterial NifS-like proteins of group I, such as IscS from *Escherichia coli* and *Azotobacter vinelandii*, have been implicated as housekeeping enzymes in Fe–S formation and are present in operons together with scaffolding proteins such as IscU and IscA (Lill and Kispal 2000; Frazzon et al. 2002). The physiological role of group-II NifS-like proteins, such as *E. coli* SufS/CsdB, in Fe–S synthesis is less evident, but work with double mutants indicates a partially overlapping function of IscS and

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SufS/CsdB in *E. coli* (Takahashi and Tokumoto 2002; Outten et al. 2004). Besides their function in Fe–S formation, NifS-like proteins of either group may be involved in the biosynthesis of thiamine, biotin, molybdenum cofactor, and seleno-protein and Se-tRNA synthesis (Mihara and Esaki 2002).

Since cysteine is the sulfur source for Fe–S formation in ferredoxin, a plastidic NifS-like protein or a similar enzyme should be involved in Fe–S formation in chloroplasts (Merchant and Dreyfuss 1998) but so far no direct evidence has been provided for this activity. Two genes encoding NifS-like proteins have been identified in the *Arabidopsis* genome. One of the encoded proteins is present in mitochondria (Kushnir et al. 2001) and the other one, called CpNifS, is located in plastids (Leon et al. 2002; Pilon-Smits et al. 2002). CpNifS belongs to group II and is able to use both Cys and SeCys as substrates, with a 300-fold lower cysteine desulfurase activity compared to its selenocysteine lyase activity (Pilon-Smits et al. 2002). In this study, we directly address the possible role of CpNifS in Fe–S cluster formation in plastids.

Materials and methods

Preparation of proteins

Arabidopsis thaliana (L.) Heynh. (ecotype Columbia) plants were grown in a growth chamber under a 14 h light/10 h dark photoperiod at 24°C/22°C. Chloroplasts were isolated from 4-week-old plants as described by Rensink et al. (1998). A stromal protein fraction was obtained from purified chloroplasts as described by Pilon-Smits et al. (2002) and stromal proteins were stored after freezing in liquid nitrogen in 0.5-ml aliquots in 50 mM Hepes–KOH (pH 8.0), 330 mM sorbitol and 12.5 mM Tris–HCl. Ultra-filtration was used to remove low-molecular-weight compounds smaller than 10 kDa from stromal protein fractions. The samples were centrifuged for 10 min at 5,000 rpm in a 10 K filter tube (Nanosep centrifugal devices; Pall Corp., Ann Arbor, MI, USA) to give a 6-fold concentration. The concentrated stromal fractions were diluted with 25 mM Tris–HCl (pH 7.5) and ultra-filtration was repeated, after which proteins were diluted in 50 mM Hepes–KOH (pH 8.0), 330 mM sorbitol and 12.5 mM Tris–HCl to the original volume.

Ferredoxin was extracted from fresh spinach that was obtained at a local health food store, essentially as described by Yocum (1982), and further purified as described by Pilon et al. (1992). The apo-fd for reconstitution assays was prepared from holo-fd by removal of the Fe–S cluster (Kato et al. 2000). Apo-fd (at 0.5 mg ml^{-1}) was stored in 100 mM Tris–HCl (pH 8.0) under nitrogen in small aliquots at −80°C. CpNifS was expressed and purified as described previously (Pilon-Smits et al. 2002).

CpNifS-specific IgY and pre-immune IgY antibodies were purified from the eggs of chickens before and after immunization with CpNifS (Pilon-Smits et al. 2002).

Reconstitution of the Fe–S cluster into apo-ferredoxin

To assay the reconstitution of the Fe–S cluster in apo-fd a modification of the method of Kato et al. (2000) was used. Apo-fd (final concentration 200 μg ml^{-1}) was mixed on ice with either purified CpNifS (5–100 μg ml^{-1}) or stromal proteins in the following solution: 50 mM Tricine–NaOH (pH 7.5), 5 mM dithiothreitol (DTT; Roche Diagnostics, Palo Alto, CA, USA), 1 mM L-cysteine (Sigma, St Louis, MO, USA), 1 mM ferrous ammonium sulfate, 20 μM PLP. The total incubation volume was 150 μl. In some experiments mentioned in Fig. 2, PLP was omitted or replaced by pyruvate. After incubation at 37°C, the reaction mixture was centrifuged at 14,000 *g* for 1 min, and directly applied to a 1-ml RESOURCE Q anion-exchange column (Amersham, Piscataway, NJ, USA) connected to a summit HPLC system with a UVD170 detector and controlled by Chromeleon software (Dionex, Sunnyvale, CA, USA). The sample loop size was 100 μl. The column had been equilibrated with 25 mM Tris–HCl (pH 7.5). The following KCl gradient was applied in this buffer at a flow rate of 1.5 ml min^{-1}: 0–4.5 min, 0 M KCl; 4.5–5 min, 0–0.25 M KCl; 5–14 min 0.25–0.55 M KCl; ramp up to 1 M KCl; 14.1–15.6 min, 1 M KCl; ramp down to 0 M KCl and hold for 2 min to re-equilibrate. Holo-fd eluted at 10.8 min. Elution was monitored by absorbance at both 280 and 420 nm. Holo-fd was quantified by signal integration at 420 nm, a characteristic absorption maximum for ferredoxin. Holo-fd (30 μg in 150 μl) was used as a standard. Background was determined in reaction mixtures that had been incubated for the same times but lacked CpNifS or stroma. Background increased with time to 4 μg holo-fd formed at 1 h and background values obtained with the same reagents on the same day were subtracted to get the reconstitution due to CpNifS activity. Low background levels required freshly prepared solutions of DTT and cysteine.

Depletion of CpNifS from stroma

In order to deplete CpNifS protein from the stroma, affinity chromatography was used. All procedures were done at 4°C. One-ml agarose beads coated with goat anti-chicken antibodies (precipHen; Aves Labs, Tigard, OR, USA) were packed into a 0.5×10 cm column and equilibrated in 50 mM Hepes–KOH (pH 8.0), 330 mM sorbitol and 12.5 mM Tris–HCl. To the column, 2 mg of IgY raised against CpNifS (Pilon-Smits et al. 2002) was applied. The flow-through was collected and re-applied twice, after which approx. 90% of IgY was bound to the column. The column was washed 5× with 6 ml buffer

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before 2 ml stroma (1 mg ml^{-1}) was applied. The flow-through was collected and re-applied four times. The final eluate was collected as stroma depleted of CpNifS.

Gel-filtration analysis

Gel filtration was done on a 1×30 cm Superdex-200 column (Pharmacia, Piscataway, NJ, USA). The column was equilibrated in 25 mM Tricine–KOH (pH 7.9), 50 mM KCl. The flow rate was 0.75 ml min^{-1} and fractions were collected every 0.5 min. A loop size of 100 µl was used. Elution was monitored by absorbance at 280 nm. The void volume was determined with blue dextran. Standards used for calibration were IgY, BSA, ovalbumin, chymotrypsinogen and RNAse. Proteins in collected fractions were concentrated by the addition of deoxycholate to 0.015% (w/v) and trichloroacetic acid to 10%, followed by centrifugation for 10 min at 12,000 *g*. Pellets were washed with ice-cold acetone, dried and resuspended in SDS–PAGE sample buffer.

General methods

Immuno-blotting procedures with CpNifS antibodies raised in chickens (IgY) were performed as described previously (Pilon-Smits et al. 2002). For quantitative detection of CpNifS the ECF staining procedure (Amersham) was used and the bands were quantified using a Storm scanner (Amersham). CpNifS activity towards L-selenocysteine was measured using 10 mM as a substrate concentration (Pilon-Smits et al. 2002). Protein was assayed according to Bradford (1976). Statistical analysis was performed with the JMP-IN software package (SAS institute, Cary, NC, USA).

Results

Ferredoxin reconstitution by pure CpNifS

To test the activity of CpNifS in Fe–S cluster formation in ferredoxin, an in vitro reconstitution assay was developed. In this assay, apo-fd was reconstituted to the holo-form by acquiring an Fe–S cluster, which was synthesized in vitro from cysteine sulfur and a ferrous iron salt. Holo-fd was separated from apo-fd and other proteins and quantified by HPLC using an ion-exchange column (see Fig. 1a). Purified CpNifS was active in stimulating holo-fd formation without involvement of any other enzyme. This reaction required the presence of the substrate cysteine and DTT (not shown). The amount of reconstituted ferredoxin was dependent on the CpNifS concentration (Fig. 1b). CpNifS is a homodimer of 43-kDa subunits and the molecular mass of spinach ferredoxin is approx. 11 kDa. Based on these data and Fig. 1, it can be calculated that for the 60-min time point at a CpNifS concentration of 10 µg ml^{-1} approx. 16 molecules of apo-fd were reconstituted per CpNifS monomer. Thus CpNifS has a catalytic role in Fe–S cluster formation in ferredoxin in vitro. Reconstitution reached a maximum after about 90 min at which time up to 80% of apo-fd was converted to holo-fd (not shown). The 30-min time point was used for all further experiments.

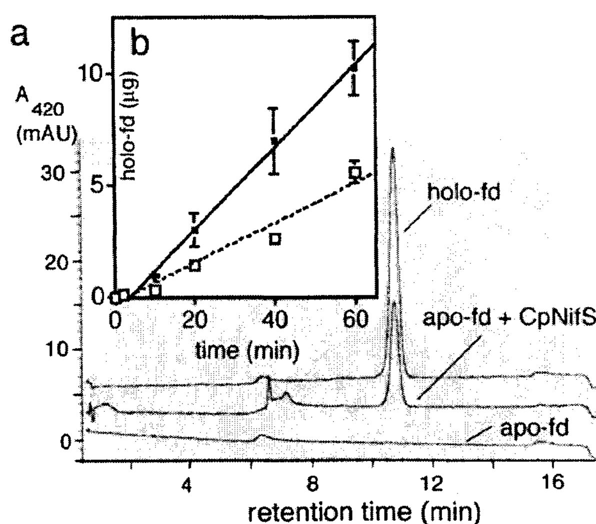

Fig. 1a, b Purified CpNifS promotes Fe–S insertion into apo-ferredoxin. **a** HPLC analysis of cysteine-dependent CpNifS-catalyzed holo-fd formation from apo-fd. Elution was monitored by absorption at 420 nm. For holo-fd and apo-fd, 30 µg protein was injected. For the trace-labeled apo-fd + CpNifS, 30 µg apo-fd was injected after 30 min incubation in a complete reconstitution assay with 7.5 µg CpNifS. **b** Holo-fd formation was measured as a function of incubation time in reconstitution reactions containing 10 µg ml^{-1} CpNifS (*open squares*) or 50 µg ml^{-1} CpNifS (*closed squares*). Data are the means ± SE of 3 experiments

To test if the activity of CpNifS in reconstitution requires its activity as a cysteine desulfurase, reactions without the cofactor PLP present were assayed. As a cofactor, PLP is stably bound to CpNifS but an inhibitive trans-amination of the cofactor is known to occur as a possible side reaction of both the cysteine desulfurase and selenocysteine lyase reactions, where pyruvate is released instead of alanine (Mihara et al. 2000). The enzyme activity can be restored by either adding fresh cofactor PLP or pyruvate, and therefore these chemicals stimulate NifS activities. Compared to the enzyme without added cofactor both PLP and pyruvate activated CpNifS in the reconstitution assay (Fig. 2a).

The stimulation of Fe–S formation observed with pyruvate was statistically significant ($P < 0.05$), while the stimulation with PLP was not. A similar pattern of stimulation was found for the selenocysteine lyase activity of CpNifS (Fig. 2b). The combination of PLP and pyruvate did not show additional improvement (not shown), suggesting that these two metabolites stimulated CpNifS activities via a similar mechanism. We conclude that CpNifS stimulates the reconstitution of ferredoxin via its cysteine desulfurase activity.

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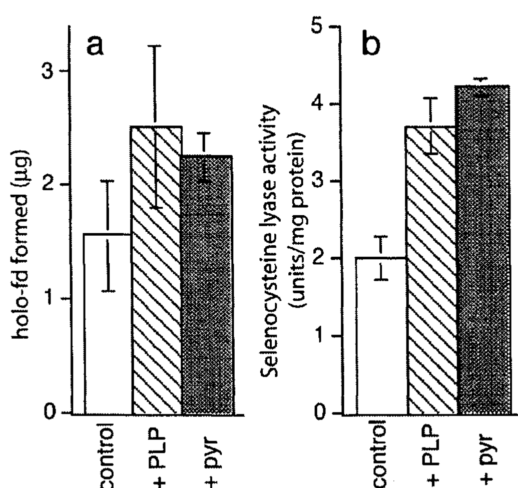

Fig. 2a, b PLP-cofactor dependence of Fe–S insertion and selenocysteine lyase activity. The Fe–S reconstitution activity (**a**) and selenocysteine lyase activity (**b**) were determined for purified CpNifS either without PLP and pyruvate (*control*), in the presence of 20 µM PLP (+ *PLP*) or with 1 mM pyruvate (+ *pyr*). CpNifS was present at 10 µg ml^{-1} in the Fe–S reconstitution assay. Data are the means ± SE of 3 experiments

Ferredoxin reconstitution by stromal proteins

CpNifS is active in the chloroplast stroma (Leon et al. 2002; Pilon-Smits et al. 2002). We compared the activities of total stromal proteins with purified CpNifS in the spinach ferredoxin reconstitution assay (Fig. 3). Since ferredoxin constitutes much less than 1% of total stromal protein, the endogenous *Arabidopsis* ferredoxin was not detected by absorption at 420 nm in our HPLC runs (not shown). Stromal proteins at 300 µg ml^{-1} showed activity comparable to 10 µg ml^{-1} CpNifS whereas 800 µg ml^{-1} stromal protein was slightly less active than

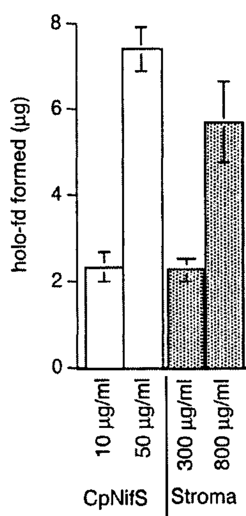

Fig. 3 Fe–S reconstitution activities of purified CpNifS and stroma. Proteins were present at the indicated concentrations. Data are the means ± SE of 3 experiments

50 µg ml^{-1} pure CpNifS (Fig. 3). Stromal proteins that were treated by ultra-filtration to remove compounds smaller than 10 kDa had a reconstitution activity comparable to untreated stroma (not shown). In order to compare the reconstitution activity between pure CpNifS and stroma, the amount of CpNifS in the stroma was quantified by Western blotting (Fig. 4a,b). A standard curve was made with pure CpNifS and the band intensities were quantified and compared with the CpNifS band present in stromal proteins. Based on this quantification we calculated that CpNifS constitutes 0.06±0.02% of total stromal protein. Based on these estimates and the data in Fig. 3 the apparent reconstitution activity of the stroma was 50–80 times more than that of pure CpNifS protein.

The reconstitution activity of the stroma requires CpNifS

To investigate whether the Fe–S cluster reconstitution activity of the stroma was dependent on CpNifS, an affinity column with CpNifS-specific IgY bound to it was used to deplete stroma of CpNifS, the removal of which was confirmed by immunoblot (Fig. 4a). Both the original stroma and the antibody-treated stroma were examined for ferredoxin reconstitution activity (Fig. 4c). The activity of the antibody-treated stroma was reduced to background levels, suggesting that the reconstitution activity of the stroma was entirely dependent on CpNifS. To further test this assumption a second depletion experiment was performed in which the depleted CpNifS protein was added back. In the second experiment, depletion was not complete as some CpNifS was still detectable by immunoblot (not shown). Nevertheless the Fe–S reconstitution activity was reduced by 75% (Fig. 4d). Importantly, adding back pure CpNifS to depleted stroma to its original concentration (180 ng ml^{-1} in the assay) restored the reconstitution activity (Fig. 4d). Stroma that had been treated with pre-immune IgY did not lose its Fe–S reconstitution activity (not shown). Together these experiments strongly indicate that CpNifS is required for Fe–S cluster formation in ferredoxin.

Since the reconstitution activity of the stroma was 50–80 times higher than that of pure CpNifS protein, other compounds in the stroma appear to activate CpNifS in vivo. To investigate whether CpNifS may be complexed to other stromal proteins, a gel-filtration experiment was performed using a high-resolution column, and the elution of CpNifS followed using immunoblotting. Three experiments were performed and representative data are given in Fig. 5. Interestingly, the CpNifS present in the stroma eluted in two peaks (Fig. 5): approx. 90% eluted as a CpNifS dimer of 86 kDa, as was observed earlier using pure CpNifS (Pilon-Smits et al. 2002). An additional, smaller amount of CpNifS eluted at a high molecular weight of approx. 600 kDa. This result indicates that CpNifS present in the

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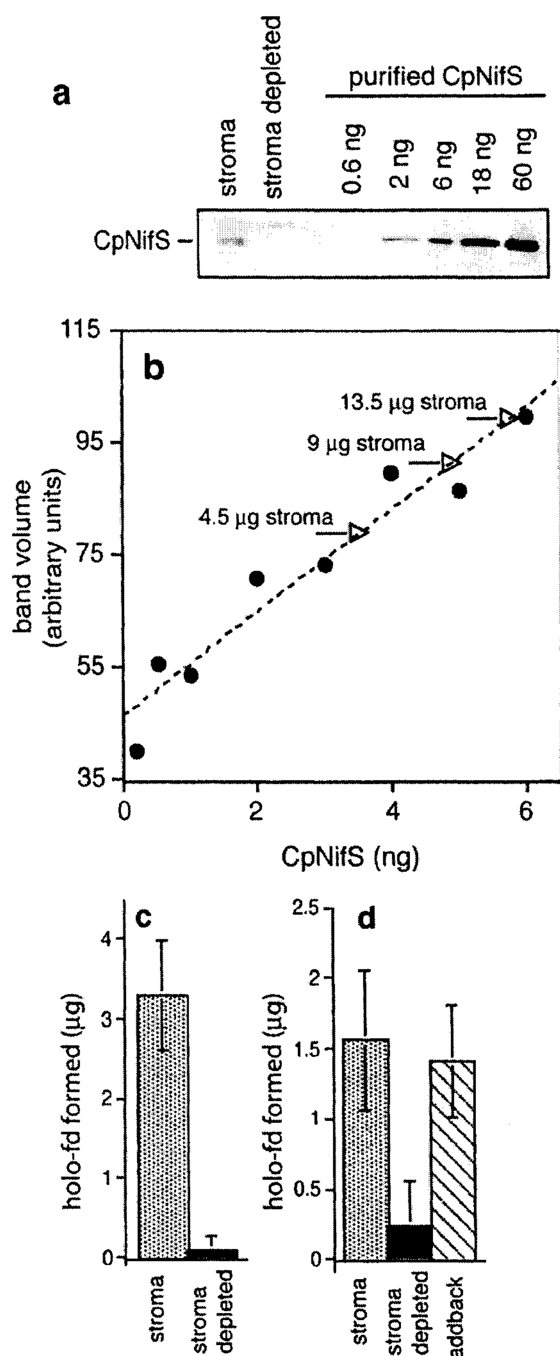

Fig. 4a–d CpNifS is required for the Fe–S reconstitution activity of stroma. **a** detection of CpNifS in untreated and depleted stroma. **b** quantitation of CpNifS in stroma. **c** Fe–S reconstitution activity of untreated and CpNifS-depleted stroma; the final stromal protein concentration was 500 µg ml^{-1}. **d** Fe–S reconstitution activity of untreated and CpNifS-depleted stroma and depleted stroma with CpNifS added back (*addback*); the final stromal protein concentration was 300 µg ml^{-1} and CpNifS was added to 180 ng ml^{-1}. Data are the means ± SE of 3 experiments

stroma interacts with other proteins in vivo and may form a transient complex with them. In contrast, purified CpNifS eluted from the column in a single peak, detected by both $A_{280\ nm}$ and Western blotting (Fig. 5b) with a retention time expected for the dimer, as was found before (Pilon-Smits et al. 2002). For purified CpNifS, no protein was detected by Western blotting at earlier retention times, fractions 1–21 (not shown).

Discussion

Iron–sulfur (Fe–S) clusters are pivotal to photosynthesis. Whereas the biogenesis of Fe–S clusters is now well studied in microbial organisms and mitochondria, still much is to be learned about Fe–S formation in chloroplasts. Whereas it was clear that cysteine provides the S for Fe–S biogenesis, the enzymatic activity responsible for the release of sulfur had not been identified thus far. In the present study it was found that the chloroplast NifS-like protein is a required component of the Fe–S machinery in plastids.

Purified CpNifS stimulates Fe–S cluster reconstitution in apo-fd. Since the Fe–S reconstitution activity requires an intact cofactor and the substrate cysteine, it depends on the catalytic action of the enzyme. This was further confirmed by results obtained with a point mutant in which the catalytic site Cys418 was replaced by Ser (our unpublished data).

The DTT required in this assay may have had two functions. First, the DTT may aid in the release of sulfane sulfur from the catalytic cysteine, making it available for incorporation in Fe–S. Second, it may help keep the cysteines on apo-fd reduced or aid in the transient formation of an Fe–S cluster and facilitate its incorporation into apo-fd. In vivo such roles may be fulfilled by glutathione and possible stromal scaffold and chaperone proteins.

Purified stroma is highly active in the reconstitution reaction and CpNifS is a required component of the stroma. When the specific activities of stromal CpNifS and pure CpNifS are compared it is evident that stromal CpNifS is more active than purified protein. Since purified CpNifS, when added to depleted stroma, has an activity comparable to native CpNifS in untreated stroma, we must conclude that the stroma contains an activity that stimulates the Fe–S cluster formation.

Arabidopsis chloroplast stroma is a highly complex mixture, containing CpNifS (Leon et al. 2002; Pilon-Smits et al. 2002) and various plastid proteins, as well as metabolites. Ultra-filtration experiments indicate that low-molecular-weight compounds are not required for optimal Fe–S reconstitution activity of stroma. It is therefore likely, that the increased activity of CpNifS was due to its interaction with other stromal proteins. Indeed, from gel-filtration analysis a fraction of the CpNifS in the stroma appears to be present as part of a complex of approx. 600 kDa. It is likely that this complex contains additional proteins that work together with CpNifS in Fe–S cluster formation in vivo. Based on what is known about microbial Fe–S assembly we can speculate about the nature of the other proteins involved.

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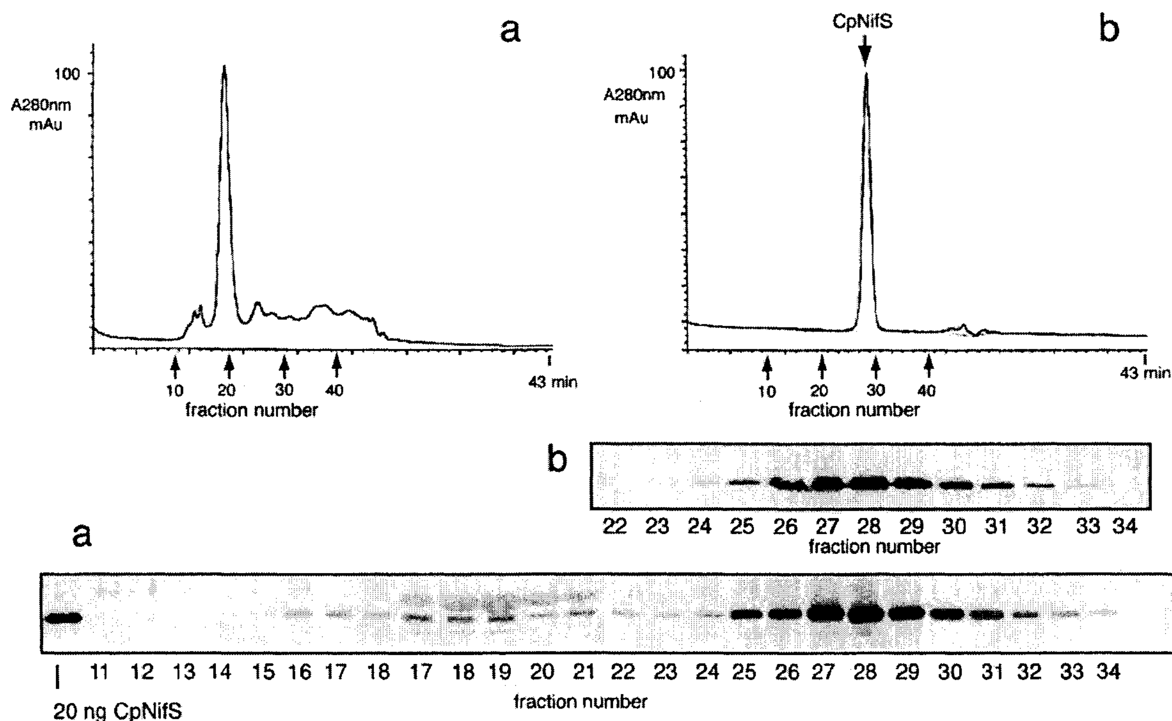

Fig. 5a, b CpNifS in the stroma is part of a high-molecular-weight complex. Proteins were separated on a Superdex S200 gel-filtration column. Elution was monitored by the absorbance at 280 nm and by immunoblotting of collected fractions with CpNifS-specific antibodies. **a** Stromal proteins (150 μg); **b** purified CpNifS (100 μg). Note: the non-specific band with a lower mobility than CpNifS in fractions 17–21 of **a** is the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase

In bacteria, the NifS-like proteins may form complexes with other factors such as NifU/IscU and NifA/IscA/SufA-like proteins in order to facilitate sulfur transfer and assembly of Fe–S clusters (Lill and Kispal 2000; Frazzon et al. 2002; Mihara and Esaki 2002). Candidates for such interacting proteins are the three Nfu proteins of chloroplasts (Leon et al. 2003; Yabe et al. 2004), which have similarity to the C-terminal domain of NifU from *Azotobacter vinelandii*, or a chloroplast-localized IscA/SufA like protein (our unpublished data). In addition, other factors with similarity to bacterial *suf* operon-encoded proteins could be involved. One protein is SufE, a small protein forming a dimer of about 35 kDa, which was shown to stimulate SufA cysteine desulfurase activity 50-fold (Loiseau et al. 2003; Ollagnier-de-Choudens et al. 2003).

Other candidate proteins are putative homologs of the bacterial SufB, C and D (Outten et al. 2003). Because of the large estimated size of the complex detected in the stroma it seems that multiple proteins may interact with CpNifS. Furthermore, the observation that the Fe–S reconstitution activity of depleted stroma was fully rescued by the addition of purified CpNifS argues that if CpNifS interacts with other proteins, such interactions are transient. The nature of these activating factors and their interaction with CpNifS will be the topic of future research.

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References

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248–254

Frazzon J, Fick JR, Dean DR (2002) Biosynthesis of iron–sulphur clusters is a complex and highly conserved process. Biochem Soc Trans 30:680–685

Kato S, Mihara H, Kurihara T, Yoshimura T, Esaki N (2000) Gene cloning, purification, and characterization of two cyanobacterial NifS homologs driving iron–sulfur cluster formation. Biosci Biotechnol Biochem 64:2412–2419

Kispal G, Csere P, Prohl C, Lill R (1999) The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. EMBO J 18:3981–3989

Kushnir S, Babiychuk E, Storozhenko S, Davey MW, Papenbrock J, De Rycke R, Engler G, Stephan UW, Lange H, Kispal G, Lill R, Van Montagu M (2001) A mutation of the mitochondrial ABC transporter Sta1 leads to dwarfism and chlorosis in the *Arabidopsis* mutant *starik*. Plant Cell 13:89–100

Leon S, Touraine B, Briat JF, Lobreaux S (2002) The AtNFS2 gene from *Arabidopsis thaliana* encodes a NifS-like plastidial cysteine desulphurase. Biochem J 366:557–564

Leon S, Touraine B, Ribot C, Briat JF, Loberaux S (2003) Iron–sulphur cluster assembly in plants: distinct NFU proteins in mitochondria and plastids from *Arabidopsis thaliana*. Biochem J 371:823–830

Li HM, Theg SM, Bauerle CM, Keegstra K (1990) Metal-ion-center assembly of ferredoxin and plastocyanin in isolated chloroplasts. Proc Natl Acad Sci USA 87:6748–6752

Lill R, Kispal G (2000) Maturation of cellular Fe–S proteins: an essential function of mitochondria. Trends Biochem Sci 25:352–356

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Loiseau L, Ollagnier-de-Choudens S, Nachin L, Fontecave M, Barras F (2003) Biogenesis of Fe–S cluster by the bacterial Suf system: SufS and SufE form a new type of cysteine desulfurase. J Biol Chem 278:38352–38359

Merchant S, Dreyfuss BW (1998) Posttranslational assembly of photosynthetic metalloproteins. Annu Rev Plant Physiol Plant Mol Biol 49:25–51

Mihara H, Esaki N (2002) Bacterial cysteine desulfurases: their function and mechanisms. Appl Microbiol Biotechnol 60:12–23

Mihara H, Kurihara T, Yoshimura T, Soda K, Esaki N (1997) Cysteine sulfinate desulfinase, a NIFS-like protein of *Escherichia coli* with selenocysteine lyase and cysteine desulfurase activities. Gene cloning, purification, and characterization of a novel pyridoxal enzyme. J Biol Chem 272:22417–22424

Mihara H, Kurihara T, Yoshimura T, Esaki N (2000) Kinetic and mutational studies of three NifS homologs from *Escherichia coli*: mechanistic difference between L-cysteine desulfurase and L-selenocysteine lyase reactions. J Biochem Tokyo 127:559–567

Ollagnier-de-Choudens S, Lascoux D, Loiseau L, Barras F, Forest E, Fontecave M (2003) Mechanistic studies of the SufS-SufE cysteine desulfurase: evidence for sulfur transfer from SufS to SufE. FEBS Lett 555:263–267

Outten FW, Wood MJ, Munoz FM, Storz G (2003) The SufE protein and the SufBCD complex enhance SufS cysteine desulfurase activity as part of a sulfur transfer pathway for Fe–S cluster assembly in *Escherichia coli*. J Biol Chem 278:45713–45719

Outten FW, Djaman O, Storz G (2004) The Suf operon requirement for Fe–S cluster assembly during iron starvation in *Escherichia coli*. Mol Microbiol 52:861–872

Pilon M, Rietveld AG, Weisbeek PJ, Kruijff B (1992) Secondary structure and folding of a functional chloroplast precursor protein. J Biol Chem 267:19907–19913

Pilon M, America T, van't Hof R, de Kruijff B, Weisbeek P (1995) Protein translocation into chloroplasts. In: Rothman SS (ed) Advances in molecular and cell biology. Membrane protein transport. JAI Press, Greenwich, vol 4, pp 229–255

Pilon-Smits EAH, Garifullina G, Abdel-Ghany S, Kato S, Mihara H, Hale K, Burkhead J, Esaki N, Kurihara T, Pilon M (2002) Characterization of a NifS-like chloroplast protein from *Arabidopsis*: implications for its role in sulfur and selenium metabolism. Plant Physiol 130:1309–1318

Raven JA, Evans MCW, Korb RE (1999) The role of trace metals in photosynthetic electron transport in O_2-evolving organisms. Photosynth Res 60:111–149

Rensink WA, Pilon M, Weisbeek P (1998) Domains of a transit sequence required for in vivo import in *Arabidopsis* chloroplasts. Plant Physiol 118:691–699

Takahashi Y, Tokumoto U (2002) A third bacterial system for the assembly of iron–sulfur clusters with homologs in archaea and plastids. J Biol Chem 277:28380–28383

Takahashi Y, Mitsui A, Hase T, Matsubara H (1986) Formation of the iron sulfur cluster of ferredoxin in isolated chloroplasts. Proc Natl Acad Sci USA 83:2434–2437

Takahashi Y, Mitsui A, Matsubara H (1990) Formation of the Fe–S cluster of ferredoxin in lysed spinach chloroplasts. Plant Physiol 95:97–103

Yabe T, Morimoto K, Kikuchi S, Nishio K, Terashima I, Nakai M (2004) The *Arabidopsis* chloroplastic NifU-like protein CnfU, which can act as an iron–sulfur cluster scaffold protein, is required for biogenesis of ferredoxin and photosystem I. Plant Cell 16:993–1007

Yocum CF (1982) Purification of ferredoxin and plastocyanin. In: Edelman M, Hallick RB, Chua N-H (eds) Methods in chloroplast molecular biology. Elsevier/N. Holland, Amsterdam, pp 973–981

Zheng L, White RH, Cash VL, Jack RF, Dean DR (1993) Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. Proc Natl Acad Sci USA 90:2754–2758

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Chapter 3

In this Chapter a CpSufE was first identified and characterized. It was found that the CpSufE activates 40-fold CysD activity of CpNifS and increases substrate affinity to cysteine while it inhibits its selenocysteine lyase activity. It stimulates 20-fold [Fe-S] cluster synthesis. Thus CpSufE seems to be an important activator as well as a regulator of CysD activity of CpNifS for [Fe-S] cluster biogenesis in chloroplasts. All the cloning, protein purification and biochemical assays were done by Hong Ye.

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Chapter 3

CpSufE Activates the Cysteine Desulfurase CpNifS for Chloroplastic Fe-S cluster formation

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SUMMARY

Plant chloroplasts synthesize their own iron sulfur clusters. CpNifS is the chloroplast cysteine desulfurase required to supply sulfur for iron-sulfur cluster biogenesis in *Arabidopsis* chloroplasts. Here we report a novel protein, CpSufE, that interacts with CpNifS. CpSufE contains a C-terminal BolA-like domain and thus is distinct from bacterial SufE proteins. CpSufE is targeted to the chloroplast stroma, indicated by GFP-localization and immunoblot experiments. Like CpNifS, CpSufE is expressed in all major tissues, with higher expression in green parts. CpSufE expression is light-dependent and regulated at the mRNA level. The addition of purified recombinant CpSufE increased the V_{max} for the cysteine desulfurase activity of CpNifS over 40 fold, and decreased the K_M toward cysteine from 0.1 to 0.043 mM. In contrast, CpSufE addition decreased the affinity of CpNifS for selenocysteine, as

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indicated by an increase in the K_M from 2.9 to 4.17 mM, and it decreased the V_{max} for selenocysteine lyase activity by 30%. CpSufE formed a complex with CpNifS, indicated by affinity chromatography and gelfiltration experiments. The iron-sulfur cluster reconstitution activity of the CpNifS-CpSufE complex toward apo-ferredoxin was 20-fold higher than that of CpNifS alone. A mutant of CpSufE in which the single cysteine was changed to serine was not active in stimulating CpNifS, although it did bind and compete with WT CpSufE. We conclude that CpNifS and CpSufE together form a cysteine desulfurase required for iron-sulfur cluster formation in chloroplasts. These results shed light on the formation of chloroplast iron-sulfur clusters, a process that is so far little understood despite its vital importance for photosynthesis, the process that determines crop productivity and drives life on earth.

INTRODUCTION

Iron-sulfur (Fe-S) cluster proteins perform a variety of biological roles in electron-transfer, catalysis, gene-regulation, and sensing of iron and oxygen (Beinert et al., 1997). Iron-sulfur cluster proteins are particularly important for photosynthesis. Measurements of metal ions in *Arabidopsis thaliana* have indicated that about 70% of the Fe in green tissue is present in chloroplasts and 40% is found in the thylakoids (Shikanai et al., 2003). Estimates in other plants indicate that up to 90% of the Fe in leaves may be present in the chloroplasts (Terry and Abadia, 1986). Within the thylakoids, the majority of the Fe is found in Fe-S cluster proteins that function in photosynthetic electron transport (Raven et al., 1999). Next to photosynthetic carbon fixation, other pivotal plastid functions that require Fe-S clusters include nitrogen

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assimilation, sulfur assimilation and pigment synthesis (for a review see: Balk and Lobreaux, 2005). All plastid types contain a number of important Fe-S cluster proteins, but the green chloroplasts particularly need to synthesize and maintain a variety of Fe-S proteins with at least 5 different cluster types (Balk and Lobreaux, 2005).

In bacteria, three separate Fe-S formation machineries have been characterized (for a review see: Johnson et al., 2005). All systems include a NifS-like cysteine (Cys) desulfurase protein, which catalyzes the conversion of cysteine to alanine and elemental sulfur or the conversion of selenocysteine (Secys) to alanine and elemental Se. Every Fe-S machinery also has scaffold proteins thought to function in the pre-assembly of clusters before transfer to target proteins. The first discovered Fe-S assembly machinery was the *Nif* system of *Azotobacter vinelandii,* which is responsible for the formation of Fe-S clusters for nitrogenase (Zheng et al., 1993). The second machinery was the *Isc* system first discovered in *A. vinelandii* and later in *Escherichia coli,* which has a housekeeping function in the formation of other cellular Fe-S proteins (Zheng et al., 1998). Mitochondrial Fe-S assembly systems in eukaryotes are remarkably similar to this *Isc* system (Lill and Kispal, 2000; Johnson et al., 2005). The third machinery was the *suf* system of *E. coli* and *Erwinia chrysanthemi,* which appears to be responsible for the formation of Fe-S clusters under oxidative stress and iron limitation (Takahashi and Tokumoto, 2002; Nachin et al., 2003; Outten et al., 2004). Based on sequence similarities several of the plastid Fe-S biosynthesis components tentatively identified to date are most related to the bacterial *suf* cluster genes; other components are unique however (Balk and Lobreaux, 2005).

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The presence of supersaturated amounts of oxygen in green tissues provides a challenge for the synthesis and maintenance of plastid Fe-S cluster proteins because of the sensitivity of these clusters to oxygen (Beinert et al., 1997). Therefore, it can be anticipated that next to the synthesis of new clusters, chloroplasts must have mechanisms to replace or repair oxidatively-damaged clusters. Chloroplasts have their own Fe-S assembly system (Takahashi et al., 1986; 1990). Fe-S cluster assembly into radiolabeled freshly imported ferredoxin precursor was demonstrated using isolated intact chloroplasts (Li et al., 1990). The reaction proceeds in the absence of cytosol (Pilon et al., 1995). Characterization of the chloroplastic Fe-S formation machinery started with the identification of a Cys desulfurase CpNifS (Leon et al., 2002; Pilon-Smits et al., 2002) and of scaffold proteins CpNfu2 (Leon et al., 2003; Touraine et al., 2004; Yabe et al., 2004) and CpIscA (Abdel-Ghany et al., 2005). CpNifS is the Cys desulfurase that converts cysteine into alanine and elemental sulfur for Fe-S formation. CpNfu2 can hold a transient Fe-S cluster. Insertion mutants in the CpNfu2 gene have a dwarf phenotype and are deficient in 2Fe-2S and 4Fe-4S proteins (Touraine et al., 2004; Yabe et al., 2004). CpIscA is a putative alternative scaffold that can accept a 2Fe-2S cluster from CpNifS, which can be transferred to apo-ferredoxin (apofd) in vitro (Abdel-Ghany et al., 2005). In addition, other Suf-type system components (Möller et al., 2001; Xu and Möller, 2004; Xu et al., 2005) and HCF101 (Lezhneva et al., 2004) may assist the Fe-S formation in plastids. The CpSufBCD complex is an ATPase and may be involved in providing ferrous iron, or in transferring the Fe-S cluster from the scaffold protein to the target protein. HCF101

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(high chlorophyll fluorescence 101) encodes a NifH-related P-loop ATPase that seems to be required for 4Fe-4S but not 2Fe-2S assembly in chloroplasts.

Since cysteine was identified as the sulfur source for Fe-S formation in chloroplasts (Takahashi et al., 1986; 1990), the Cys desulfurase activity of CpNifS is likely essential for Fe-S formation in chloroplasts. Indeed, the depletion of CpNifS led to the loss of Fe-S reconstitution activity of chloroplast stroma (Ye et al., 2005). This Cys desulfurase of chloroplasts is distinct from the Cys desulfurases NifS and IscS of the Nif and Isc type assembly systems and is more similar in sequence to SufS (Pilon-Smits et al., 2002). *In vitro*, the purified CpNifS has a much lower Cys desulfurase activity than Secys lyase activity (Pilon-Smits et al., 2002). However, the Fe-S cluster reconstitution activity of CpNifS in stroma is 50-80 fold higher than that of CpNifS alone, suggesting some factors - most likely proteins - are activating the Cys desulfurase activity of CpNifS (Ye et al., 2005). The activation mechanism has been unclear until the characterization of CpSufE in this study. CpSufE is the latest component of the Suf-type Fe-S formation system identified in *Arabidopsis* chloroplasts. We show here that CpSufE forms a complex with CpNifS, thus stimulating Cys desulfurase activity over 40-fold, and enhancing CpNifS-dependent Fe-S reconstitution *in vitro*.

MATERIALS AND METHODS

Cloning and plasmid construction - The *A. thaliana* CpSufE coding sequence was amplified by PCR using cDNA as a template. Copy-DNA was prepared from DNase-treated total RNA prepared from two-week old seedlings as described (Pilon-

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Smits et al, 2002). Primers used for CpSufE amplification were *SufE-precursor* and *SufE-Bam* (Table 1). The PCR product was digested with *Nco*I and *BamH*I and then ligated into vector pET11d (Novagen, Madison, WI), digested with the same restriction enzymes to produce plasmid pPrSufE. To subclone the mature sequence of CpSufE in pET28a (Novagen, Madison, WI) for expression as a His_6-tagged protein, PCR was performed with a set of nested primers, *SufE-mature* and *SufE-Bam* (Table 1). Plasmid pPrSufE was used as a template. The PCR product was digested with *Nde*I and *BamH*I and subcloned in vector pET28a, which was digested with the same enzymes to produce plasmid pMSufE.

To change the single cysteine in the mature sequence of CpSufE to serine, recombinant PCR was performed. In the first round, two fragments were amplified with primer set *T7* and $SufE_{C65S}$*-R*, and primer set *T7 terminator* and $SufE_{C65S}$*-F*, respectively (Table 1). pMSufE was used as template. The two products from the first round of PCR were together used as template for the second round of PCR, with primers *SufE-mature* and *SufE-Bam* (Table 1). The resulting PCR product was digested with *Nde*I and *BamH*I and subcloned in vector pET28a to produce plasmid $pMSufE_{C65S}$. For GFP localization, the transit peptide coding sequence of CpSufE was amplified with flanking primers *SufE-GFP-F* and *SufE-GFP- RT* (Table 1), while the full-length protein sequence was amplified with flanking primers *SufE-GFP-F* and *SufE-GFP-R* (Table 1). The pPrSufE plasmid was used as a template. PCR products were digested with *Sal*I and *Nco*I, and inserted into the *Sal*I/*Nco*I digested GFP reporter plasmid 35Ω-SGFP(S65T) (Miras et al., 2002) to create the plasmids TP/SufE-GFP and Full length/SufE-GFP, respectively.

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For site-directed mutagenesis of $cysteine_{388}$ in mature CpNifS to serine, a strategy was used similar to the one described above for the CpSufE mutant. In the first round of PCR, two fragments were amplified with primers *T7* and *$NifS_{C388S}$-R*, and primers *T7 terminator* and *$NifS_{C388S}$-F* respectively (Table 1), using pMNFS-8 (Pilon-Smits et al., 2002) as a template. In the second round of PCR the two fragments were fused together and amplified with primers NFS-mat and NFS-B2 (Table 1). The final PCR product was digested with *Nco*I and *BamH*I, and ligated into vector pET11d, resulting in $pMNFS_{C388S}$. All constructs were verified by DNA sequencing. The plasmids used for protoplast transformation were prepared using the Plasmid Midi Kit (Qiagen, Valencia, CA).

Sequence analysis and alignments - Sequence analysis was performed using the Mac Vector sequence analysis software (International Biotechnologies, New Haven, CT). Searches for sequence similarity were performed using the BLAST network service provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Sequence alignment was performed using ClustalW at European Bioinformatics Institute, ExPASY Proteomics tools (http://www.ebi.ac.uk/clustalw).

Preparation of proteins - For overexpression of CpSufE, *E.coli* BL21 (DE3) $codon^{+}$ (Stratagene, La Jolla, CA) was transformed with plasmid pMSufE or with $pMSufE_{C65S}$ for the mutant protein. Two liter of LB medium containing 50 μg ml^{-1} kanamycin was inoculated with 1/100 volume of overnight culture. Cells were grown at 37 °C to an OD_{600} of 0.5, and expression was induced by addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) followed by incubation for 3 h at 37°C.

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The culture was chilled on ice, and the cells were collected by centrifugation for 5 min at 5,000 *g* at 4°C. From here on, all procedures were performed at 4 °C except where mentioned. The bacterial pellet was washed with 150 mM NaCl and re-suspended in 50 mM Tris-HCl, pH 7.5, then passed twice through a French press (8,000 p.s.i) to disrupt the cells. The lysate was centrifuged for 20 min at 12,500 x g and the cleared supernatant was loaded at a flow rate of 3 ml min^{-1} onto His-Bind iminodiacetic acid (IDA) agarose (Novagen, Madison, WI) in a 1.6 x 20 cm column, which was saturated with $NiSO_4$, washed and equilibrated in 50 mM Tris-HCl, pH 7.5. The column was washed with four volumes of 50 mM Tris-HCl, pH 7.5; followed by 4 volumes of 1 M NaCl, 50 mM Tris-HCl pH 7.5; 6 volumes of 50 mM Tris-HCl, pH 7.5 again and two volumes of 0.1 M imidazole in 50 mM Tris-HCl, pH 7.5, respectively. His_6-tagged CpSufE was eluted with 4 volumes of 1 M imidazole in 50 mM Tris-HCl, pH 7.5. Fractions of 6 ml were collected. Peak fractions were pooled and dialyzed overnight against 25 mM Tris-HCl, pH 7.5. Pure His-tagged CpSufE ran as a single band on SDS-PAGE after staining with Coomassie Brilliant Blue. To produce cleaved CpSufE, the pooled peak fractions were dialyzed overnight against 20 mM Tris/HCl, pH 8.4, followed by incubation with thrombin in a 1:1000 w/w ratio (thrombin : target protein) at 4 °C for 8 h in 20 mM Tris/HCl, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$ as suggested by the manufacturer (Novagen, Madison, WI). The cleavage mixture was subsequently applied to a 10 x 1 cm Resource-Q column, (Amersham Biosciences, Piscataway, NJ) equilibrated in 25 mM Tris/HCl, pH 8.0, at room temperature and connected to a Summit HPLC system (Dionex, Sunnyvale, CA). The column was eluted with a linear gradient from 0 to 1 M NaCl in 25 mM Tris-HCl, pH 8.0, and fractions of 2 ml were

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collected. Elution was monitored by detection of the OD at 280 nm and 220 nm. The purified cleaved CpSufE was dialyzed overnight against 25 mM Tris/HCl, pH 7.5, and stored frozen at -80 °C before use in activity assays. Typical yields were 5-10 mg l^{-1} of culture. The purified protein migrated as a single band on SDS-PAGE (12.5% gel) and ran as a single peak in analytical HPLC runs on a 1 ml Resource Q column (Amersham Biosciences, Piscataway, NJ).

The His6-tagged and cleaved $CpSufE_{C65S}$ were purified essentially as the WT CpSufE protein. WT CpNifS protein was prepared as described before (Pilon-Smits et al., 2002). For overexpression of $CpNifS_{C388S}$, *E.coli* BL21 (DE3) $codon^+$ (Stratagene, La Jolla, CA) was transformed with plasmid $pMNFS_{C388S}$. The recombinant $CpNifS_{C388S}$ was prepared essentially as WT CpNifS (Pilon-Smits et al., 2002). Purified $CpNifS_{C388S}$ was eluted from a calibrated 1 x 30 cm Superdex-S200 gel-filtration column (Amersham Biosciences, Piscataway, NJ) at the same retention time as the WT protein, and a native molecular mass of 83 kDa was calculated, suggesting that $CpNifS_{C388S}$ is a dimer like the wild type protein. Holo- and apo-Fd were prepared as described (Ye et al., 2005).

Enzyme assays - Cys desulfurase activity was assayed at 25^0C essentially as described (Outten et al., 2003). Briefly, 160μl reaction mixture contained 25 mM Tris-HCl pH 7.4, 100 mM NaCl, 2.5μM enzyme (0.11 mg/ml CpNifS, 0.09 mg/ml CpSufE), 10 μM pyridoxal 5' phosphate (PLP, Acros Organics, Morris Plains, NJ), 1 mM DTT (Roche, Mannheim, Germany), and 500 μM cysteine (Sigma, St. Louis, MO). The reaction was stopped by addition of 20 μl 20 mM N, N dimethyl-p-phenylenediamine in 7.2 M HCl. Methylene blue was formed by addition of 20 μl 30

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mM $FeCl_3$ in 1.2 M HCl and was assayed by measuring the absorbance at 670 nm. The Secys lyase activity was measured as described (Pilon-Smits et al., 2002). One unit of enzyme activity corresponds to 1 micromole substrate converted min^{-1}. To estimate kinetic constants for both the Cys desulfurase and Secys lyase activities of CpNifS the reaction velocities were measured over a wide range of substrate concentrations (0.01 – 20 mM). The data in Michaelis-Menten plots were fitted by an iterative method to estimate K_M and V_{max} values, using the software program Enzfitter (Biosoft, Cambridge, UK). The Fe-S reconstitution assay was performed as described before (Ye et al., 2005). For all statistical analyses, the JMP-IN software (SAS institute, Cary, NC) was used.

Protein coelution experiments – 100 μg His-tagged CpSufE (WT or mutant) and 100 μg CpNifS (WT or mutant) were mixed at room temperature, and loaded on a 0.5 ml His-Bind iminodiacetic acid (IDA) agarose column (Novagen, Madison, WI). The column was washed with 2 ml of 50 mM Tris-HCl, pH 7.5, and subsequently with 2 ml of 1 M NaCl, 50 mM Tris-HCl pH 7.5, followed by 2 ml of 50 mM Tris-HCl, pH 7.5 again, and 1 ml of 50 mM Tris-HCl pH 7.5, 0.1 M imidazole. Finally, the column was eluted with 2 ml of 1 M imidazole, 50 mM Tris-HCl pH 7.5, and one ml fractions were collected. Eluted proteins were analyzed by SDS-PAGE.

Gelfiltration – Sizes of protein complexes were estimated by gelfiltration experiments (as described before by Ye et al., 2005) using a 1x30 cm Superdex-200 column (Pharmacia, Piscataway, NJ), which was connected to a summit HPLC system with a UVD170 detector and controlled by Chromeleon software (Dionex, Sunnyvale, CA). The loop size was 0.2 ml. The column was equilibrated in 25 mM Tricine-KOH,

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pH 7.9, 50 mM KCl. The flow rate was 0.75 ml min^{-1} and fractions were collected every 0.5 min. Elution was monitored by absorbance at both 280 and 220 nm and by immunoblotting of collected fractions. The void volume was determined with blue dextran. Standards used for calibration were IgY, BSA, ovalbumin, chymotrypsinogen and RNAse.

Plant sampling – Arabidopsis thaliana (Ecotype Columbia-0) plants were grown on soil with supplementary light on a 15h-light/9h-dark cycle for 4 weeks. Total leaf homogenate, chloroplast stroma, and RNA from different tissues were prepared as described (Pilon-Smits et al., 2002). For light regulation analysis of CpSufE, Arabidopsis plants were grown on half-strength Murashige and Skoog (MS) agar medium (Murashige and Skoog, 1962) for 2 weeks, either on a 15h-light/9h-dark cycle or in complete darkness. Protein and RNA preparations from total leaf homogenate were described before (Pilon-Smits et al., 2002).

Sub-cellular localization of GFP-fusion proteins - The plasmids TP/SufE-GFP or Full length/SufE-GFP were transformed into Arabidopsis protoplasts and expressed proteins were observed under a confocal microscope, as described (Abdel-Ghany et al., 2005).

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Antibodies and immunoblotting - Cleaved CpSufE in 100 mM NaCl, 25 mM sodium phosphate, pH 7.5 was used to raise polyclonal antibody in rabbits at a commercial facility (PRF&L, Canadensis, PA). The CpNifS antibodies have been described (Pilon-Smits et al., 2002). The Hsp70 antibody was purchased from Sigma (St. Louis, MO). RuBisCo antibody was purchased from AgriSera (Vannas, Sweden). Immunoblotting was performed as described (Pilon-Smits et al., 2002).

RNA blot analysis – Total RNA from different Arabidopsis tissues was prepared, electrophoresed, and probed with a ^{32}P-labeled 900 bp CpSufE cDNA, essentially as described before (Pilon-Smits et al., 2002).

RESULTS

Identification of CpSufE and sequence characteristics – CpNifS is a Cys desulfurase (Leon et al., 2002; Pilon-Smits et al., 2002) required for iron sulfur cluster formation in the chloroplast (Ye et al., 2005). However, compared to most Cys desulfurases with a housekeeping role in Fe-S cluster formation, the Cys desulfurase activity of purified CpNifS is very low, despite a very high selenocysteine lysase activity (Pilon-Smits et al., 2002). When the *in vitro* reconstitution activity of purified CpNifS protein was compared to the activity of CpNifS in stromal extracts it was concluded that CpNifS in stroma is about 80 times more active (Ye et al., 2005). Thus a stimulatory activity must be present in plastids. We considered that this stimulatory activity could at least in part be explained if plastids contain a homologue of SufE, a protein which stimulates the Cys desulfurase activity of the bacterial SufS proteins. A

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SufE-like sequence (At4g26500) was identified by a BLAST search within the *Arabidopsis* genome database (TAIR; http:// www.arabidopsis.org), using the *E.coli* SufE (Outten et al., 2003) as a query sequence. The genomic sequence of At4g26500 contains a single predicted exon. A cDNA containing the full coding sequence was obtained by RT-PCR. Sequence analysis of the cDNA confirmed the presence of a single uninterrupted coding sequence in the genome (not shown). The open reading frame is predicted to encode a full-length precursor protein of 371 amino acids (see Fig. 1A), including a putative chloroplast targeting peptide (66 amino acids) as predicted by the TargetP program (www.expasy.org, and see Emanuelsson et al., 2000). Because of the predicted location and similarity with SufE proteins we named At4g26500 CpSufE, for chloroplastic SufE. The predicted mature polypeptide is 305 amino acids long, with a molecular weight of 33.6 kDa and an isoelectric point of 4.87. The mature size of the Arabidopsis CpSufE homologue is much larger than what was reported for the bacterial SufE proteins, which are about 15 kDa in size (Loiseau et al., 2003; Outten et al., 2003).

A sequence alignment was performed to determine the similarity of the putative *Arabidopsis* CpSufE with possible SufE homologues from various organisms, as shown in Figure 1. The predicted cleavable chloroplast targeting sequence of the Arabidopsis protein is underlined. A similar N-terminal extension, predicted to be a transit sequence by the TargetP program, is also found in the rice (*Oryza sativa*) protein but is not found in the prokaryotic homologues, as expected. All SufE homologues show sequence conservation in the mature protein domain, including a highly conserved single cysteine that was shown to be critical for function in the

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bacterial proteins (Ollagnier-de-Choudens et al. 2003; Outten et al. 2003). This SufE domain region corresponds to the N-terminal half of the predicted mature proteins in Arabidopsis and rice. In this region the plant proteins are most related in sequence to the cyanobacterial SufE. For example, the sequence motif GCVSQV, which includes the conserved cys, is conserved between *Arabidopsis* CpSufE, rice and cyanobacterial SufE but different in the other bacterial proteins. The two plant proteins are predicted to have a C-terminal domain of about 150 amino acids, which is absent from the prokaryotic homologues. The last 88 residues of this plant-specific C-terminal protein domain show very good sequence similarity to *E. coli* BolA (45% identity and 61% similarity). BolA of *E. coli* may function in the control of cell shape in response to nutrition (Santos et al., 2002). BolA-like proteins are found ubiquitously but so far a molecular function has not been identified for these proteins (Santos et al., 2002). The rice and Arabidopsis proteins display good sequence conservation over their entire length (42% identity), but the conservation in the BolA-like domain is even higher, with 72% identity.

Localization of CpSufE in chloroplasts - The TargetP program predicted a cleavable transit sequence of 66 amino acids and a chloroplast localization of CpSufE. To examine the sub-cellular localization, we constructed fusions with the green fluorescent protein (GFP). In the first construct, the N-terminus of GFP was fused to the sequence encoding the CpSufE transit peptide; in a second construct the N-terminus of GFP was fused to the C-terminus of the full-length precursor CpSufE. *GFP* alone expressed from the same constitutive promoter was used as a control. Constructs were separately introduced into *Arabidopsis* protoplasts and the

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localization in cells was analyzed using confocal laser microscopy. Fluorescence corresponding to GFP expressed without a transit sequence was excluded from the chloroplasts as expected (Fig. 2A, upper panel). In contrast, green fluorescence from the transit sequence fusion was localized to the chloroplast stroma, as indicated by the overlay of green fluorescence and red auto-fluorescence from chlorophyll (Fig. 2A, middle panel). Green fluorescence from the full-length CpSufE coupled to GFP was localized to discrete locations in the chloroplasts (Fig. 2A, lower panel).

The localization of CpSufE in chloroplast stroma was verified by immunoblotting (Fig. 2B). CpSufE antibody detected as little as 0.5 ng purified CpSufE. The antibody detected a protein of 37 kDa in total leaf homogenate (TH) and this signal was quantitatively recovered in the chloroplast stroma fraction (St), (Fig 2B). The pre-immune serum at the same dilution did not recognize any bands in these samples (data not shown). These data indicate the presence of CpSufE in stroma. Based on the band intensities, we estimate that the amount of CpSufE present in 20 μg of stromal protein is between 10 and 20 ng. Thus the abundance of CpSufE in stroma is between 0.05 and 0.1 % of total protein, similar to the abundance (0.06%) reported for CpNifS (Ye et al., 2005). In conclusion, the immunoblotting and GFP experiments strongly suggest a stromal localization of CpSufE.

Expression analysis of CpSufE – Expression patterns can give clues about the function of a protein. To determine the CpSufE mRNA and protein expression patterns in different *Arabidopsis* tissues we performed RNA blot and immunoblot analyses (Fig. 3A). The RNA blot analysis revealed that CpSufE is expressed in all tested tissues with slightly higher expression levels in green photosynthetic tissues

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(leaves and stems) than non-green tissues (roots and flowers). Immunoblots confirmed this expression pattern at the protein level and showed that CpSufE and CpNifS have comparable expression patterns. The presence of CpSufE in plastids and the elevated expression in green tissues are typical for proteins involved in photosynthesis. Many photosynthesis-related genes are regulated by light. We therefore investigated if CpSufE expression is influenced by light. CpSufE protein was found to be expressed at about 3-fold lower levels in plants grown in the dark, compared to light-grown plants (Fig. 3B) and this can be ascribed to lower expression of the CpSufE mRNA in the dark (Fig. 3C). However, even though there was less expression of CpSufE in the dark there was still significant expression, and the effect of light on the expression of the large subunit of RuBisCo, a known light-induced chloroplast protein involved exclusively in photosynthesis was larger in comparison.

CpSufE stimulates Cys desulfurase activity and changes the substrate affinity of CpNifS – To allow us to investigate the activity of CpSufE, we purified the mature-sized protein. The N-terminus of the protein was fused to a six-histidine tag and a thrombin cleavage site (Fig. 4A) for efficient purification by immobilized metal ion affinity chromatography (Fig. 4B). To remove the His6 tag, the protein was incubated with thrombin at 4°C until the His6 tag was completely cleaved as judged by SDS-PAGE, and CpSufE was purified from the mixture by HPLC using a Resource-Q anion exchange column (Fig. 4B). The purified CpSufE was a colorless protein with a M_w of about 40 kDa as judged by SDS-PAGE (Fig. 4B), a slightly slower migration than what is expected based on the theoretical Mw of 33.6 kDa. However, CpSufE is rather acidic and this may well influence the mobility on SDS-PAGE. N-terminal

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sequence analysis yielded the sequence GSHMASS, which indicated that the protein was correctly cleaved and purified. The cleaved CpSufE was used for all biochemical characterizations, unless indicated otherwise.

CpSufE alone did not show any activity in either the Cys desulfurase assay (Fig. 5A) or the Secys lyase assay (Fig. 5C), both of which are activities displayed by CpNifS. To test if CpSufE changes the kinetic properties of CpNifS, the activities of CpNifS alone and of CpNifS plus CpSufE in a 1:1 molar ratio were assayed over a concentration range for both substrates (Table II). CpNifS alone displayed a barely detectable level of Cys desulfurase activity (V_{max} = 0.0013 µmol/min/mg) but displayed high Secys lyase activity (V_{max} = 2.44 µmol/min/mg), in agreement with our previous results (Pilon-Smits et al., 2002). However when CpSufE was added to CpNifS in a 1:1 molar ratio, we observed a 40-fold increase in the V_{max} for Cys desulfurase activity compared to CpNifS alone. In contrast, the V_{max} for Secys lyase activity was reduced by 30%. The addition of CpSufE to CpNifS caused the K_m for cysteine to decrease 2.5-fold. On the other hand, the K_M for selenocysteine increased slightly (1.4-fold). Titration experiments at saturating substrate concentration in which the amount of CpNifS was kept constant while CpSufE was varied indicated that the Cys desulfurase activity of CpNifS depended on the amount of CpSufE present. The stimulation by CpSufE reached an apparent saturation point at a CpSufE: CpNifS molar ratio of 5:1, at which point we observed a 60-fold stimulation (Fig. 5A).

CpNifS has 5 cysteines, one of which, Cys_{388}, is conserved and predicted to be required for cys desulfurase activity (Pilon-Smits et al., 2002) based on similarity with bacterial CsdB (Mihara et al., 2000) for which a structure is published (Fujii et

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al., 2000). To investigate if this cysteine in CpNifS is required for the activity of the protein we purified a mutant in which the cysteine was altered to serine. Purified mutant $CpNifS_{C388S}$ displayed the same absorbance spectrum and elution profile from a gelfiltration column as the WT protein. Similar to what had been found for the CsdB Cys-mutant (Mihara et al., 2000), the mutant $CpNifS_{C388S}$ protein retained about 80% of its Secys lyase activity (Fig. 5B). This indicates that under the assay conditions the decomposition of selenocysteine does not depend on the active site cysteine and that the mutant $CpNifS_{C388S}$ enzyme was folded and active. However, the $CpNifS_{C388S}$ mutant protein had no detectable Cys desulfurase activity anymore, neither alone nor with CpSufE present. Thus the conserved $cysteine_{388}$ is essential for Cys desulfurase activity of CpNifS, but not for Secys lyase activity.

CpSufE has a single conserved cysteine, Cys_{65}, in its mature sequence. To test the requirement of the thiol group for CpSufE activity we expressed and purified a mutant in which the cys was changed to serine. Like the wild-type CpSufE, the mutant $CpSufE_{C65S}$ did not show activity by itself (Fig. 5 B and C). Only WT CpSufE but not $CpSufE_{C65S}$ displayed stimulation of CpNifS cys desulfurase activity, indicating that the conserved $cysteine_{65}$ is essential for this function (Fig. 5C). Both CpSufE and $CpSufE_{C65S}$ had a similar small but noticeable negative effect on the Secys lyase activity of CpNifS.

CpSufE forms a complex with CpNifS – The observed alterations of the catalytic properties of CpNifS by CpSufE prompted us to investigate if CpSufE and CpNifS form a complex. To address this question, we analyzed co-elution in metal chelate affinity chromatography and gelfiltration experiments. For the first type of

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experiment we used the His_6-tagged CpSufE and incubated the protein with an equal amount of CpNifS before loading onto a Ni-IDA column. The column was treated with a series of salt washes before elution with 1 M imidazole. Samples of the loaded and imidazole-eluted proteins were analyzed by SDS-PAGE (Fig. 6A). Wild-type CpNifS was found to co-elute with the His-tagged wild-type CpSufE (Fig. 6A). Control experiments showed that CpNifS did not bind to the nickel column by itself (data not shown). Remarkably, the same elution pattern was found for CpNifS and His-tagged CpSufE regardless whether the proteins had the wild-type sequences or were mutated at the cysteine residues (Fig. 6A). Thus, CpNifS and CpSufE interact and this interaction does not require the conserved Cys residues in either protein, which are however needed for cys desulfurase activity. Another chloroplast protein, CpIscA, which may serve as a chloroplast scaffold protein for Fe-S assembly (Abdel-Ghany et al., 2005), did not co-elute with CpSufE or with CpSufE and CpNifS (data not shown).

The interaction of CpSufE and CpNifS was further investigated by gelfiltration experiments in which untagged protein was used (Fig. 6B). The native molecular weight of recombinant CpSufE and of the $CpSufE_{C65S}$ mutant was determined by comparing the elution time from a Superdex 200 gelfiltration column with standards. In gelfiltration experiments, CpSufE eluted in a single, symmetrical, peak at 18.5 min (Fig. 6B, trace 1). The same elution profile was seen for $CpSufE_{C65S}$ (not shown). The retention time of 18.5 min corresponds to an estimated M_w of ~ 70 kDa, indicating that purified recombinant CpSufE and the $CpSufE_{C65S}$ mutant are homodimeric proteins. CpNifS eluted as a single peak at 17.6 min (Fig. 6B, trace 2),

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which corresponds to a size of 86 kDa, in agreement with the mass expected for homodimeric CpNifS (Pilon-Smits et al., 2002). When WT CpSufE and CpNifS (WT) were mixed in a 1:1 molar ratio, and applied to the gel-filtration column, we found most of the protein to elute in a symmetric peak with a retention time of 17.2 min (Fig. 6B, trace 3). Comparison to standards indicated an apparent M_w of ~110 kDa, indicating the formation of a complex that is bigger than the added M_w of two monomers but smaller than the added M_w of two dimers. Both CpSufE and CpNifS were found to be present in the peak as judged from SDS-PAGE electrophoresis (Fig. 6C). When the mutated $CpSufE_{C65S}$ was incubated with CpNifS and loaded onto the gelfiltration column an asymmetric peak with a retention time of 16.5 min was observed (Fig. 6B, trace 4). These data indicate that the mutant CpSufE retains CpNifS-binding ability, but in this case the complex has an apparent size of ~150 kDa, which is in good agreement with the cumulative M_w of a CpSufE dimer and a CpNifS dimer. When the cysteine mutant of CpNifS was substituted for the wild-type protein and incubated with CpSufE, the same elution pattern was obtained that was observed with WT CpNifS (data not shown).

To analyze the native molecular weight of CpSufE in chloroplast stroma, stromal proteins were separated by gelfiltration. Fractions were collected, concentrated by TCA precipitation and analyzed by immunoblotting (Fig. 6D). The majority of CpSufE eluted in fractions corresponding in apparent size to a dimeric form of CpSufE (fractions 28-32). However, a significant amount of CpSufE was detected in earlier fractions indicating that at least some CpSufE was in higher molecular weight complexes in the stroma.

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The Cys$_{65}$Ser mutation in CpSufE is dominant negative in vitro – As shown above, the mutant CpSufE (CpSufE$_{C65S}$) in which the active center cysteine is changed to serine, was not active in stimulating the Cys desulfurase activity of CpNifS, but it did bind to CpNifS. To test if this binding inhibits the activation of CpNifS by WT CpSufE, the three proteins were incubated with the CpSufE$_{C65S}$ in 5-fold excess, and assayed for Cys desulfurase activity (Fig. 7). The presence of CpSufE$_{C65S}$ severely inhibited the stimulatory activation of CpNifS by WT CpSufE, presumably due to competition for binding to CpNifS. This competitive inhibition was dependent on the molar ratio of WT and mutant CpSufE, since an equal amount of mutant CpSufE$_{C65S}$ roughly inhibited 50% of the activity of WT CpSufE (data not shown). Therefore, the interaction of mutant CpSufE$_{C65S}$ with CpNifS had a dominant negative effect on the activity of WT CpSufE *in vitro*. The simplest explanation for this result is the sequestration of CpNifS into an inactive complex by the mutant CpSufE. The mutant CpNifS (CpNifS$_{C388S}$) in which the active center cysteine is changed to serine was not active as a Cys desulfurase, but it did still bind to CpSufE (Fig. 6). However, an excess of CpNifS$_{C388S}$ did not inhibit the activation of WT CpNifS by CpSufE (Fig. 7) indicating a significant mechanistic difference in the interaction of mutant CpNifS with WT CpSufE as compared to the interaction of the WT CpNifS and mutant CpSufE.

CpSufE enhances the Fe-S reconstitution in ferredoxin 20-fold – To quantitatively measure Fe-S cluster formation, a Fe-S reconstitution assay for ferredoxin was used (Ye et al., 2005). The effect of CpSufE on the Fe-S cluster formation in ferredoxin was tested in these CpNifS-dependent reconstitution assays

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(Fig. 8). CpSufE by itself did not mediate any Fe-S cluster formation, while CpNifS only displayed a very low level of activity. However, the mixture of 5 μg CpNifS and 4 μg CpSufE (~1:1 molar ratio) reconstituted ~21 μg ferredoxin, equivalent to the activity of 100 μg pure CpNifS. This indicated a 20-fold enhancement of CpNifS-dependent Fe-S cluster formation by CpSufE.

DISCUSSION

The Cys desulfurase activity of CpNifS is required for Fe-S formation in chloroplast stroma (Ye et al., 2005). However, purified CpNifS has only a very low level of Cys desulfurase activity by itself (Pilon-Smits et al, 2002). In *in vitro* experiments, the newly identified chloroplast protein CpSufE interacts with CpNifS and stimulates the Cys desulfurase activity of CpNifS over 40-fold. Purified CpSufE enhances the Cys desulfurase activity of CpNifS by affecting both the substrate affinity and V_{max}. Furthermore, the CpNifS-dependent Fe-S reconstitution activity was found to be enhanced 20-fold by CpSufE. These findings provide at least a partial explanation for the 50 – 80-fold higher reconstitution ability of chloroplast stroma compared to CpNifS alone (Ye et al., 2005) and directly connect a SufE-like protein with Fe-S cluster formation. CpSufE and CpNifS should also be able to interact *in vivo* because both proteins are localized in the stroma and have comparable expression patterns and expression levels. Thus, CpSufE appears to be an important regulator for both Cys desulfurase activity and Fe-S formation in plastids.

SufE proteins are evolutionary conserved. Homologues are found in a wide variety of organisms including prokaryotes and eukaryotes. All these proteins have a

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conserved Cys residue that seems to function in a sulfur transfer pathway, accepting the S atom that is bound as sulfane sulfur to an active site Cys in a type II, SufS-like Cys desulfurase (Ollagnier-de Choudens et al., 2003; Outten et al., 2003). This mechanism seems to be conserved in the chloroplast and the cys residue of CpSufE was also found to be essential for efficient Cys desulfurase activity of CpNifS. However, compared to bacterial proteins, the plant homologues have some unique properties. The Arabidopsis and rice SufE proteins have an extra C-terminal domain in their mature proteins that shows good sequence similarity to *E. coli* BolA. The function of this BolA-like domain is at this point unclear. In *E. coli*, BolA is involved in the regulation of cell division in response to nutrition (Santos et al., 2002), however the molecular mechanism by which BolA functions is not yet resolved. One attractive possibility is that this domain is not involved in the stimulation of Cys desulfurase activity by itself but maybe serves a regulatory role. Perhaps this BolA-like domain functions as an interacting partner for other regulatory proteins that could be used to regulate CpSufE and thus Cys desulfurase activity. More recently, it is hypothesized that BolA protein is a reductase interacting with a glutaredoxin thus likely involved in oxidative stress response (Huynen et al. 2005). Regulation of the Cys desulfurase activity in the chloroplast may be a requirement, because the plastids are the major site of sulfur reduction and Cys synthesis in the plant cell. Too much Cys desulfurase activity could deplete the Cys pool and be detrimental. On the other hand, Cys desulfurase activity should be sufficient to allow Fe-S formation and repair as needed. The Secys lyase activity is not much affected by CpSufE. Therefore plastids have the ability to decompose Secys and thus prevent the unspecific and toxic incorporation of

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Se in proteins, regardless of the activity of CpSufE. Indeed, overproduction of CpNifS (without CpSufE) affected Se-tolerance but had relatively little effect on S metabolism in plants (Van Hoewyk et al, 2005).

We found that CpSufE and CpNifS interact, as measured by affinity chromatography and gel-filtration experiments. However, the observations that CpSufE is present mostly in dimeric form in the stroma and that maximum stimulation of CpNifS requires a 5-fold excess and not just an equal amount of CpSufE both may be taken to indicate that the interaction is transient. Remarkably, the mutation of the conserved cysteine to serine in either protein still allows interaction of CpNifS and CpSufE as measured by chromatography coelution, indicating that other amino acid residues are responsible for the binding. Interestingly, when present in excess the mutant $CpSufE_{C65S}$ has a dominant negative effect on the activity of CpNifS, competitively inhibiting the activation by WT CpSufE (Fig. 7). This experiment further confirms the concept that the binding and formation of a two-component Cys desulfurase is a prerequisite for CpNifS activation by CpSufE. In contrast to the dominant negative effect of the CpsufE mutant, an excessive amount of mutant $CpNifS_{C388S}$ did not prevent WT CpNifS from being activated by WT CpSufE, possibly because the $CpNifS_{C388S}$ has a lower ability to bind to CpSufE than the WT protein. These observations may be explained by the following proposed reaction mechanism: CpNifS binds the substrate cysteine. CpSufE then binds to CpNifS and the binding affinity is increased after CpNifS has decomposed the substrate Cys and has a sulfane sulfur bound to its active site - something the CpNifS mutant can not do, and therefore it does not act dominant negative. After transfer of the S to the cysteine

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of WT CpSufE the interaction becomes again reversible and CpSufE can leave and deliver the S to downstream targets such as scaffolding proteins *in vivo. In vitro* this acceptor could be the DTT in the reaction mixture. The mutant CpSufE cannot receive the S from CpNifS and therefore is not released as easily, resulting in the observed inhibitory effect.

In addition to CpSufE, homologues have been found in *Arabidopsis* for all of the bacterial Suf proteins, and all are predicted to be present in plastids. Therefore, it seems that in the course of evolution plants have retained the entire Suf machinery including SufABCDES for chloroplast Fe-S cluster assembly (for review see Balk and Lobreaux, 2005). CpNifS is the SufS-like protein, which is a Cys desulfurase supplying sulfur for the Fe-S cluster formation. CpSufE is the SufE-like protein, which is a Cys desulfurase activator as reported in this paper. CpIscA is a SufA-like scaffold protein where a transient 2Fe-2S cluster is assembled (Abdel-Ghany et al., 2005). A similar pattern of gene expression was found for these proteins, supporting the possibility that they may function in the same biochemical process of Fe-S assembly (this work; Abdel-Ghany et al., 2005). Chloroplastic SufB, SufC and SufD are homologues of bacterial SufB, SufC and SufD, respectively. They form a complex, which displays an iron-stimulated ATPase activity (Moller et al., 2001; Xu and Moller, 2004; Xu et al., 2005). However, a direct role of CpSufBCD in the Fe-S formation still needs to be demonstrated.

The Suf system in bacteria is responsible for Fe-S formation under oxidative stress and iron limitation (Outten et al., 2004). This may explain why plants contain a Suf-like system for biosynthesis of Fe-S clusters in chloroplasts, as they are oxygen-

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producing compartments. It has been suggested that the S in SufE may be somewhat shielded. Besides Suf-like proteins, chloroplasts also contain Nfu-like scaffolds and the HCF 101 protein for Fe-S cluster biosynthesis (Leon et al., 2003; Lehzneva et al., 2004), suggesting that the Fe-S cluster biogenesis machinery in chloroplasts is more complex than any single operon in bacteria. Because of the barrier formed by the envelope, Fe-S clusters synthesized outside plastids cannot be easily supplied to chloroplasts, which is self-sufficient for the synthesis of its Fe-S clusters. Since plant plastids require a variety of Fe-S cluster types and have at least 20 Fe-S proteins there is perhaps a requirement for multiple-protein components. For instance, Nfu1-3 and CpIscA may be alternative scaffolds either for assembling different types of Fe-S clusters or for different physiological conditions.

In conclusion, the results presented here reveal a new component of the iron-sulfur biogenesis machinery in plant chloroplasts. These results are of significance since little is known about how chloroplast iron-sulfur clusters are made, despite their vital importance for photosynthesis and assimilatory nitrogen and sulfur assimilation. Photosynthesis is the process that determines plant productivity and ultimately drives life on earth. Its importance for biology cannot be overstated. Also, plant iron status is of importance for human health, since billions of people worldwide are suffering from iron deficiency and most of the dietary iron in developing countries is derived from vegetarian food sources.

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REFERENCES

Abdel-Ghany, S.E., Ye, H., Garifullina, G.F., Zhang, L., Pilon-Smits, E.A.H., and Pilon, M. (2005) *Plant Physiol.* **138**, 161-172

Balk, J., and Lobreaux, S. (2005) *Trends Plant Sci.* **10**, 324-331

Beinert, H., Holm, R.H., and Munck, E. (1997) *Science* 277, 653-659

Emanuelsson, O., Nielsen, H., Brunak, S., and von Heijne, G. (2000) *J. Mol. Biol.* **300**, 1005-1016

Fujii, T., Maeda, M., Mihara, H., Kurihara, T., Esaki, N., and Hata, Y. (2000) *Biochemistry* **39**, 1263-1273

Huynen MA, Spronk CA, Gabaldon T, and Snel B. (2005) *FEBS Lett.* **579**, 591-596

Johnson, D., Dean, D.R., Smith, A.D., and Johnson, M.K. (2005) *Annu. Rev. Biochem.* **74**, 247–281

Leon, S., Touraine, B., Briat, J.F., and Lobreaux, S. (2002) *Biochem. J.* **366**, 557-564

Leon, S., Touraine, B., Ribot, C., Briat, J.F., and Lobreaux, S. (2003) *Biochem. J.* **371**, 823-830

Lezhneva, L., Amann, K., and Meurer, J. (2004) *Plant J.* **37**, 174-185

Li, H.-M., Theg, S.M., Bauerle, C.M., and Keegstra, K. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **8**, 6748-6752

Lill, R., and Kispal, G. (2000) *Trends Biochem. Sci.* **25**, 352-356

Loiseau, L., Ollagnier-de-Choudens, S., Nachin, L., Fontecave, M., and Barras, F. (2003) *J. Biol. Chem.* **278**, 38352-38359

Mihara, H., Kurihara, T., Yoshimura, T., and Esaki, N. (2000) *J. Biochem.* **127**, 559-567

Miras, S., Salvi, D., Ferro, M., Grunwald, D., Garin, J., Joyard, J., and Rolland, N. (2002) *J. Biol. Chem.* **277**, 47770-47778

Moller, G.M., Kunkel, T., and Chua, N.-H. (2001) *Genes Dev.* 15, 90-103

Murashige, T., and Skoog, F. (1962) *Physiol. Plant* **15**, 437-497

Nachin, L., Loiseau, L., Expert, D., and Barras, F. (2003) *EMBO J.* **22**, 427-437

Ollagnier-de-Choudens, S., Lascoux, D., Loiseau, L., Barras, F., Forest, E., and Fontecave, M. (2003) *FEBS Lett.* **555**, 263-267

Outten, F.W., Wood, M.J., Munoz, F.M., and Storz, G. (2003) *J. Biol. Chem.* **278**, 45713-45719

Outten, F.W., Djaman, O., and Storz, G. (2004) *Mol. Microbiol.* **52**, 861-872

Pilon, M., America, T., van 't Hof, R., de Kruijff, B., and Weisbeek, P. (1995) *Membrane protein transport*, JAI Press, Greenwich

Pilon-Smits, E.A.H., Garifullina, G.F., Abdel-Ghany, S., Kato, S., Mihara, H., Hale, K.L., Burkhead, J.L., Esaki, N., Kurihara, T., and Pilon, M. (2002) *Plant Physiol.* **130**, 1309-1318

Raven, J.A., Evans, M.C., and Korb, R.E. (1999) *Photosynthesis Res.* **60**, 111-149

Santos, J.M., Lobo, M., Matos, A.P.A., de Pedro, M.A., and Arraiana, C.M. (2002) *Mol. Microbiol.* **45**, 1729-1740

Shikanai, T., Müller-Moulé, P., Munekage, Y., Niyogi, K.K., and Pilon, M. (2003) *Plant Cell* **15**, 1333-1346

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Takahashi, Y., Mitsui, A., Hase, T., and Matsubara, H. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2434-2437
Takahashi, Y., Mitsui, A., and Matsubara, H. (1990) *Plant Physiol.* **95**, 97-103
Takahashi, Y., and Tokumoto, U. (2002) *J. Biol. Chem.* **277**, 28380 – 28383
Terry, N., and Abadia, J. (1986) *J. Plant Nutr.* **9**, 609-646
Touraine, B., Boutin, J.P., Marion-Poll, A., Briat, J.F., Peltier, G., and Lobreaux, S. (2004) *Plant J.* **40,** 101-111
Van Hoewyk, D., Garifullina, G.F., Ackley, A.R., Abdel-Ghany, S.E., Marcus, M.A., Fakra, S., Ishiyama, K., Inoue, E., Pilon, M., Takahashi, H., and Pilon-Smits, E.A.H. (2005) *Plant Physiol.* in press
Xu, X.M., and Moller, S.G. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101**, 9143-9148
Xu, X.M., Adams, S., Chua, N-H., Moller, S.G. (2005) *J. Biol. Chem.* **280**, 6648-6654
Yabe, T., Morimoto, K., Kikuchi, S., Nishio, K., Terashima, I., and Nakai, M. (2004) *Plant Cell* **16**, 993-1007
Ye, H., Garifullina, G.F., Abdel-Ghany, S.E., Zhang, L., Pilon-Smits, E.A.H., and Pilon, M. (2005) *Planta* **220,** 602-608
Zheng, L., White, R.H., Cash, V.L., Jack, R.F., and Dean, D.R. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90,** 2754-2758
Zheng, L., Cash, V.L., Flint, D.H., and Dean, D.R. (1998) *J. Biol. Chem.* **273**, 13264-13272

FOOTNOTES

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The abbreviations used are: At, Arabidopsis thaliana; Cp, chloroplast; Fd, ferredoxin; Cys, cysteine; SeCys, selenocysteine; CBB, Coomassie Brilliant Blue; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WT, wild type; M_w, molecular weight; PLP, pyridoxal 5' phosphate; DTT, dithiothreitol; RuBisCo, ribulose bisphosphate carboxylase oxygenase.

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FIGURE LEGENDS

Fig. 1. A. Sequence alignment of SufE-like proteins from various organisms. The predicted transit sequence of *Arabidopsis* CpSufE is underlined. Identical residues are shaded in gray. The conserved cysteine at position 131 in *Arabidopsis* precursor CpSufE (or position 65 in mature CpSufE) is boxed. Species used are as follows. At: *Arabidopsis thaliana*, Os: *Oryza sativa*, Sy: *Synchocystis* sp. PCC 6803, Ec: *Escherichia coli*, Er: *Erwinia chrysanthemi*, Ag: *Agrobacterium tumefaciens*.
B. Schematic domain structure of CpSufE

Fig. 2. Localization of CpSufE in chloroplasts. *A*, Subcellular localization of GFP and CpSufE-GFP fusion proteins. CpSufE-GFP fusion proteins were expressed in *Arabidopsis* protoplasts, and observed using a confocal laser-scanning microscope. Green fluorescence, red chlorophyll fluorescence and overlays of green and red signals are shown. *B,* Immunoblotting analysis of total leaf homogenate (TH) and chloroplast stroma (St). Homogenate and stroma, containing 2.5 μg chlorophyll for each, were separated by SDS-PAGE (12.5% gel), blotted to nitrocellulose after which CpSufE was detected with a specific antibody. Pure CpSufE protein served as a positive control. Large subunit of RuBisCo was detected with corresponding antibody to evaluate equal loading.

Fig. 3. Expression analysis of CpSufE. *A*, Immunoblot analysis of CpSufE and CpNifS expression in different tissues. Each fraction represents 10 μg of protein. *B,* RNA blot of CpSufE in different tissues. Ten microgram of total RNA from different tissues was

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separated by electrophoresis, transferred to hybond N^+ membrane, and probed with a ^{32}P-labeled 900 bp CpSufE cDNA (top). An ethidium bromide-stained agarose gel was used to verify equal loading (bottom). *C.* Immunoblot of CpSufE in homogenate from light/dark-grown Arabidopsis. Twenty microgram of leaf homogenate protein from light- or dark-grown Arabidopsis were separated by electrophoresis, transferred to nitrocellulose membrane, and probed with CpSufE antibody. Arabidopsis cytosolic actin Hsp 70 was used to evaluate equal loading. The large subunit of RuBisCo was used as a control of light-regulation. *D.* RNA blot of CpSufE in homogenate from light/dark-grown Arabidopsis. Ten microgram of total leaf homogenate RNA from light or dark-grown Arabidopsis was separated by electrophoresis, transferred to hybond N^+ membranes, and probed with a ^{32}P-labeled 900 bp CpSufE cDNA (top). Ethidium bromide-stained agarose gel was used to show loading (bottom).

Fig. 4. Purification of CpSufE. *A,* Expression region of CpSufE in the pET28a vector. Both nucleotide and amino acid sequences are used to show the express region of CpSufE in pET28a. The His6 tag, thrombin cleavage site, and mature CpSufE in the expressed protein are labeled. The restriction sites of *Nde*I and *Bam*HI for subcloning CpSufE are labeled in Italic font. *B.* SDS-PAGE of CpSufE at different purification stages. *lane 1,* total cell lysate of *E. coli* BL21 (DE3) / codon+ expressing His-tagged CpSufE; *lane 2,* eluate of 1 M imidazole from nickel column containing His-tagged CpSufE; *lane 3,* eluate of ~ 0.3 M NaCl in linear salt gradient from Resource-Q column after thrombin treatment. *C,* Elution profile (*A*280) of purified CpSufE from a Superdex 200 gelfiltration column.

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Fig. 5. CpSufE stimulates Cys desulfurase activity 40-fold but reduces Secys lyase activity of CpNifS. *A,* Cys desulfurase activity of CpNifS (2.5 μM) mixed with CpSufE in molar ratios ranging from 0 to 20 (CpSufE : CpNifS). *B,* Selenocysteine lyase activities of proteins and protein combinations present at 2.5 μM. *C,* Cysteine desulfurase activities of proteins and protein combinations present at 2.5 μM. Data are the means ± SE of 3 experiments.

Fig. 6. CpSufE forms a complex with CpNifS. *A,* Ni-NTA-column coelution of His-tagged CpSufE and CpNifS. 100 μg His-tagged CpSufE (WT or mutant) and 100 μg CpNifS (WT or mutant) were mixed and loaded on a 0.5 ml Ni-IDA agarose column, followed by a series of washes as described in Materials and Methods. The 1 M imidazole eluate was collected and separated on SDS-PAGE gel followed by staining with Coomassie Brilliant Blue. Purified His-tagged CpSufE, CpNifS and mutant proteins were run on the same gel as controls. *B,* Superdex-200 gelfiltration elution profiles ($A_{280\,nm}$). *trace 1,* 15 μg CpSufE; *trace 2,* 17 μg CpNifS; *trace 3,* mixture of 15 μg CpSufE and 17 μg CpNifS; *trace 4,* mixture of 15 μg $CpSufE_{C65S}$ and 17 μg CpNifS. *C,* SDS-PAGE analysis of gelfiltration fractions described in panel B. Aliquots of the indicated fractions were collected and separated on SDS-PAGE gels followed by staining with Coomassie Brilliant Blue. The peak fraction in each gelfiltration run is marked by an asterisk. *D*, Immunoblot analysis of CpSufE in gelfiltration fractions of chloroplast stroma. One milligram of stromal protein was

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separated by gelfiltration. Proteins in collected fractions were concentrated by TCA precipitation, and separated by SDS-PAGE. CpSufE was detected with specific antibody.

Fig. 7. Dominant negative effect of CpSufE$_{C65S}$. Cysteine desulfurase activity was assayed for the protein mixtures shown at the indicated protein concentrations. Data are the means $\pm$ SE of 3 experiments.

Fig. 8. CpSufE enhances the Fe-S reconstitution in ferredoxin 20-fold. Proteins were incubated with 50 mM Tricine-NaOH, pH 7.5, 5 mM DTT, 1 mM L-Cys, 1 mM ferrous ammonium sulfate, 20 μM PLP for 30 min, followed by addition of 30 μg of apo-Fd. After 20 min, the reconstituted holo-Fd was measured by HPLC. Data are the means $\pm$ SE of 3 experiments.

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Table 1. Sequence of oligonucleotides used for cloning and plasmid constructions

Oligonucleotide	5'-3' Sequences	Restriction site underlined
SufE-precursor	CATGCCATGGCAGCAGCGATGTCTTCTTC	*Nco* I
SufE-mature	GGAATTCCATATGGCTTCATCATCTCCGTCGAG	*Nde* I
SufE-Bam	CGGGATCCTCAAACCTCAGCAGGAGTCT	*Bam* HI
$SufE_{C65S}$-F	AATAAAGTAGAAGGATCTGTTTCTCAGGTTTGG	–
$SufE_{C65S}$-R	CCAAACCTGAGAAACAGATCCTTCTACTTTATT	–
T7	CGAAATTAATACGACTCACTATAG	–
T7 terminator	TGCTAGTTATTGCTCAGCGGT	–
SufE-GFP-F	GAATGGTCGACATGGCAGCAGCGATGTCTT	*Sal* I
SufE-GFP-R	CATGCCATGGCCTCAGCAGGAGTCTTTGC	*Nco* I
SufE-GFP-RT	CATGCCATGGGTAGCTTCGGTGGAAGCTCT	*Nco* I
$NifS_{C388S}$-F	AAGGTCAGGACACCACTCCGCACAGCCACTCCA	–
$NifS_{C388S}$-R	TGGAGTGGCTGTGCGGAGTGGTGTCCTGACCTT	–
NFS-mat	See (18)	
NFS-B2	See (18)	

Table 2. CpSufE changes the kinetic properties of CpNifS. K_m values and reaction velocities (V_{max}) toward cysteine and selenocysteine were determined over the range of 0.01 to 20 mM of substrate concentration, as described in the Materials and Methods. The program Enzfitter (Biosoft, Cambridge, UK) was used to calculate K_m and V_{max} values. Data are the means and standard error of 3 experiments.

Substrate	CpNifS		CpNifS + CpSufE	
	K_m (mM)	V_{max} (unit/mg)	K_m (mM)	V_{max} (unit/mg)
Cysteine	0.10±0.02	0.0013±0.0002	0.043±0.011	0.055±0.002
Selenocysteine	2.90±0.36	2.44 ±0.07	4.17 ±0.24	1.76 ±0.11

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AtSufE   MAAAMSSSCCASSLRLIPFKRTLFSSIHYPAKTLLLRPLKPSEVPSFRRTIITFQKISTG  60
OsSufE   MASAAATSSSASLRLLTKPPKPLLS------KPHLLTLCAP---VSFQR--LVARSSASP  49
SySufE   ------------------------------------------MNR---------LS  5
EcSufE   ------------------------------------------------------------
ErSufE   ------------------------------------------------------------
AgSufE   ------------------------------------------------------------

AtSufE   IVPPPSASSSPSSYGDLQPIEELPPKLQEIVKLFQSVQEPKAKYEQLMFYGKNLTPLDSQ  120
OsSufE   TPSPSAAAAAAAASGSGVDPAQLPPALRDIVALFQSVPDPRTRYKQLLAYAARLPPMDPA  109
SySufE   IIFPNLVMAN----------ATLPPNLAKIVERFQRHTDPKKRYEQLLWYGKKLEPMMEE  55
EcSufE   -------------------MALLPDKEKLLRNFLRCANWEEKYLYIIELGQRLPELRDE  40
ErSufE   -------------------MAQLPDPQKLLRNFSRCSNWEEKYLYIIELGAGLAPLSDA  40
AgSufE   -------------------MASL---ETILDDFAFLDDWEDRYRYVIELGKALPELPED  37
                                         :: *     : . :*  ::  .  *  :

AtSufE   FKTRENKVEGCVSQVWVRAFFDE---ERNVVYEADSDSVLTKGLAALLVKGLSGRPVPEI  177
OsSufE   LKTDANRVRGCVSQVWVHAAPEEGGAPGRVSFQADSDAQLTKGLAALLVLGLSGAPARDV  169
SySufE   GKIAANKVQGCVSQVYITADLED----GKVMYQGDSDAQLVKGLVALLIQGLNGLTPTEI  111
EcSufE   DKSPQNSIQGCQSQVWIVMRQN---AQGIIELQGDSDAAIVKGLIAVVFILYDQMTPQDI  97
ErSufE   QRQDGNRVSGCQSQVWIDLASN---EQGNVVLHGDSDAAIVKGLIAIVFSLYQGLSVREI  97
AgSufE   KRTPENKVQGCASQVWLVSHSDGA-EDPLMTFEGDSDAHIVRGLVAIVLTVYSGRKASEI  96
          :    *  : ** ***::     :      :   ..***: :.:** *::.    .    ::

AtSufE   LRITP-DFAVLLGLQQSLSPSRNNGLLNMLKLMQKKALHL---EVKGEEDSSSGESSESS  233
OsSufE   AMVPV-EFIELLGIRQSLSPSRNSGLLNMLSLMKRKALEVATGEVTTEEIGSQEVVQEVA  228
SySufE   VELTP-DFIEATGLQVSLTPSRANGFYNIFKMMQTKAIAFQLGQSYGEG----------  159
EcSufE   VNFDVRPWFEKMALTQHLTPSRSQGLEAMIRAIRAKAAALS-----------------  138
ErSufE   VELDVRPFFASLALTQHLTPSRSQGLEAMLRAVRARASALI-----------------  138
AgSufE   ADLDAIEVFSKIGLVEHLSAQRANGLRSMIKRIRDEARLLAA----------------  138
           .        .:    *:..*  .*:   ::   :: .*  .

AtSufE   FVSIPETKDEANVPEVDLESKPDLVEDLGTEKIDDSES---GSNVVALGSRGMRIREKLE  290
OsSufE   ERPAAKEKEPEFAAFGAREEEGSEVHSPQEEQLEEMPADVMEGNGGLGGGRQERIKESLE  288
SySufE   ------------------------------------------------------------
EcSufE   ------------------------------------------------------------
ErSufE   ------------------------------------------------------------
AgSufE   ------------------------------------------------------------

AtSufE   KELDPVELEVEDVSYQHAGHAAVRGSAGDDGETHFNLRIVSDAFQGKSLVKRHRLIYDLL  350
OsSufE   RGLSPVQLQIEDISHLHKGHAGVSGSNG---ETHFNVRVVSEAFQGKSLLKRHRAVYDLL  345
SySufE   ------------------------------------------------------------
EcSufE   ------------------------------------------------------------
ErSufE   ------------------------------------------------------------
AgSufE   ------------------------------------------------------------

AtSufE   QDELKSGLHALSIVAKTPAEV  371
OsSufE   QDELKNGLHALSIDAKTPSEV  366
SySufE   ---------------------
EcSufE   ---------------------
ErSufE   ---------------------
AgSufE   ---------------------
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B

CpSufE

Figure 1

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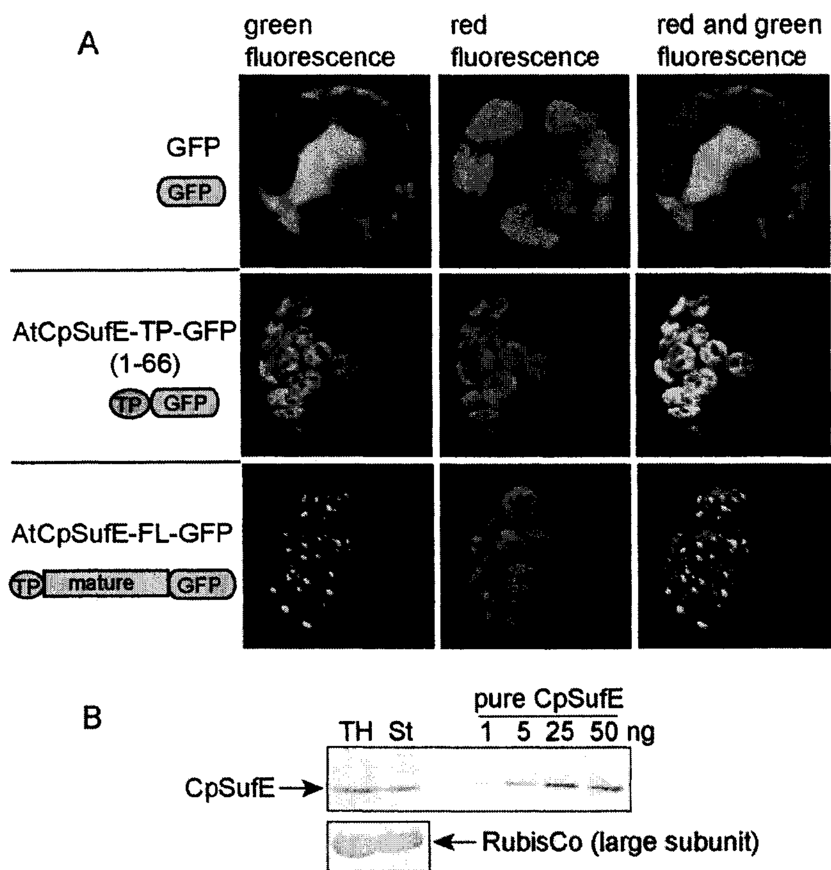

Figure 2

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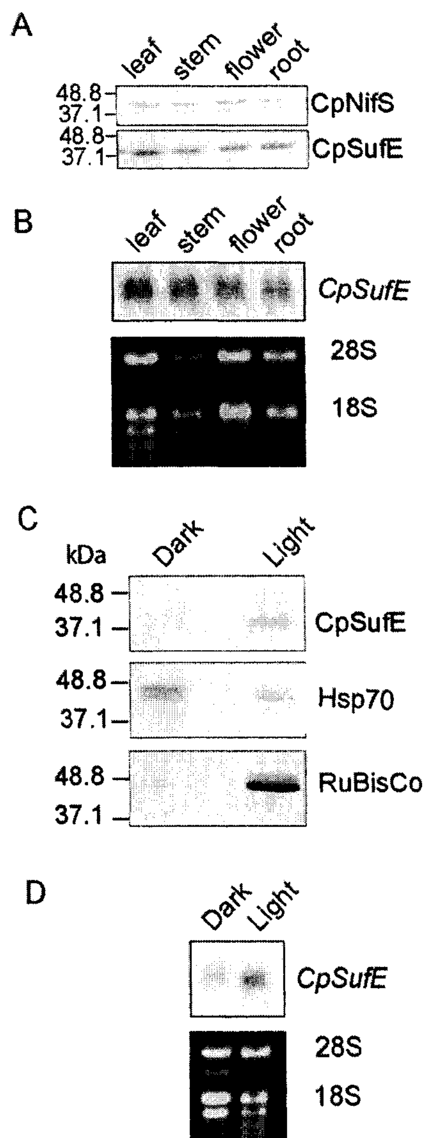

Figure 3

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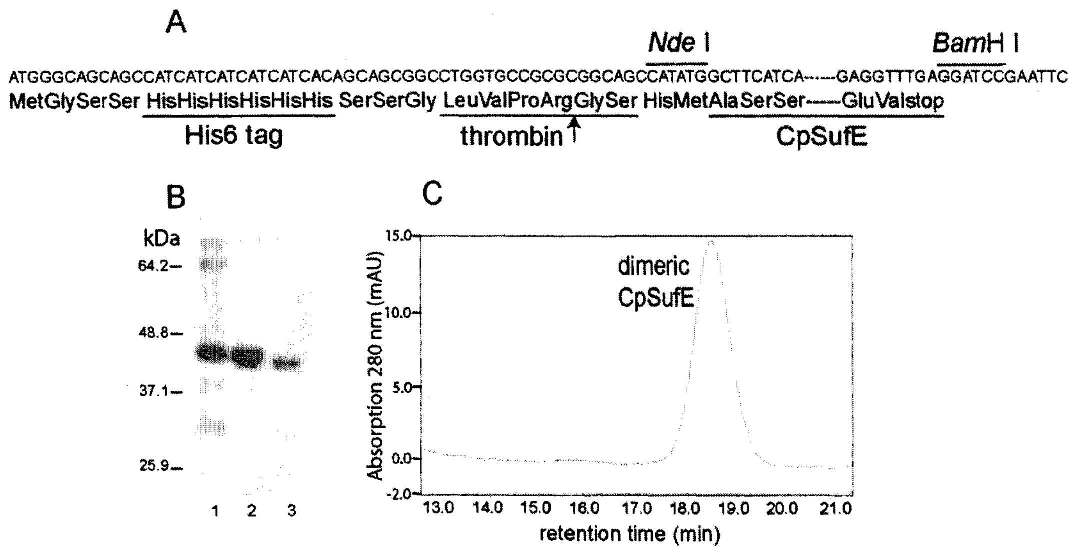

Figure 4

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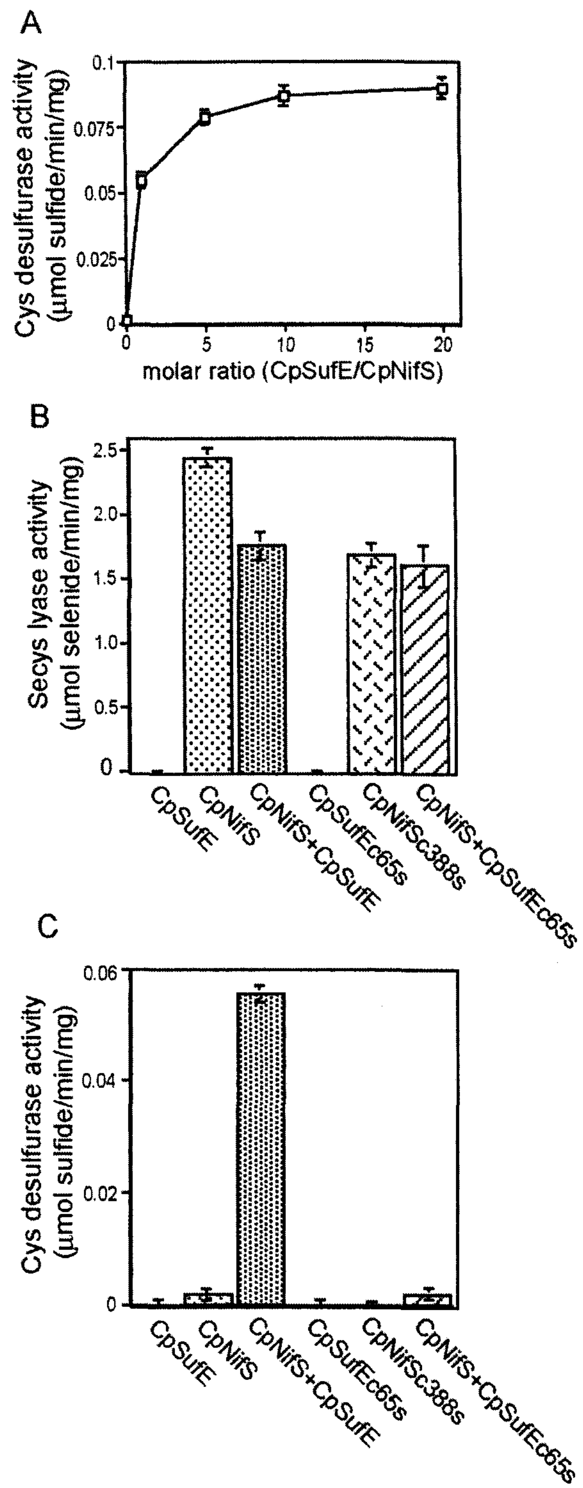

Figure 5

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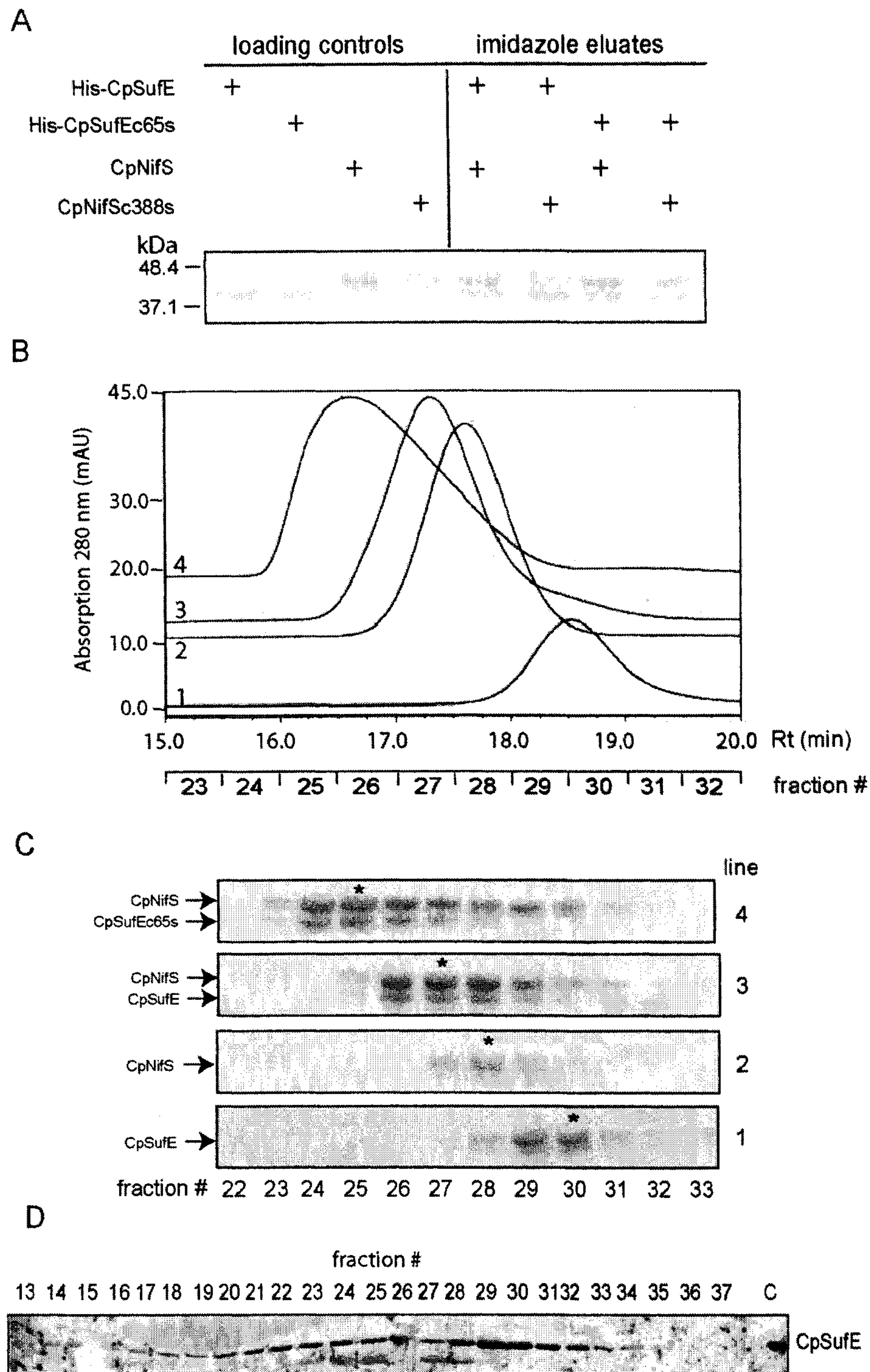

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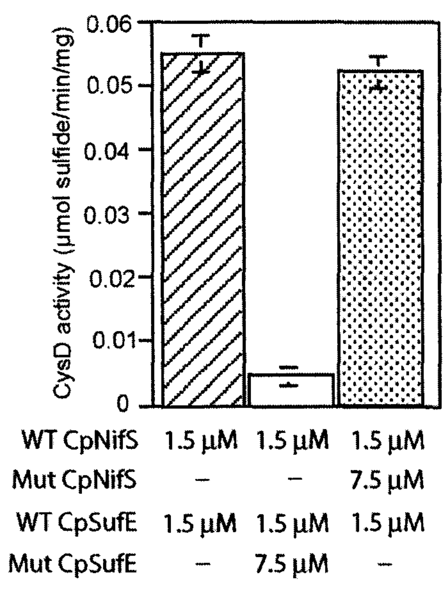

Figure 7

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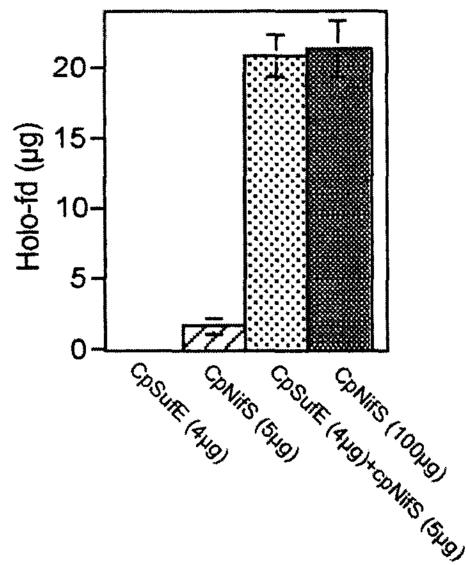

Figure 8

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Chapter 4

In this Chapter a CpIscA was identified and characterized. It was found that the CpIscA acquires an [Fe-S] cluster incubated with CpNifS in the presence of Fe and cysteine. The cluster can be transferred to apoferredoxin. Thus the CpIscA is a molecular scaffold of the [Fe-S] biogenesis machinery in chloroplasts. All the biochemical assays (in Fig. 4 panel C, and Fig. 6-8) were done by Hong Ye.

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Iron-Sulfur Cluster Biogenesis in Chloroplasts. Involvement of the Scaffold Protein CpIscA[1]

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The chloroplast contains many iron (Fe)-sulfur (S) proteins for the processes of photosynthesis and nitrogen and S assimilation. Although isolated chloroplasts are known to be able to synthesize their own Fe-S clusters, the machinery involved is largely unknown. Recently, a cysteine desulfurase was reported in Arabidopsis (*Arabidopsis thaliana*; AtCpNifS) that likely provides the S for Fe-S clusters. Here, we describe an additional putative component of the plastid Fe-S cluster assembly machinery in Arabidopsis: CpIscA, which has homology to bacterial IscA and SufA proteins that have a scaffold function during Fe-S cluster formation. CpIscA mRNA was shown to be expressed in all tissues tested, with higher expression level in green, photosynthetic tissues. The plastid localization of CpIscA was confirmed by green fluorescent protein fusions, in vitro import, and immunoblotting experiments. CpIscA was cloned and purified after expression in *Escherichia coli*. Addition of CpIscA significantly enhanced CpNifS-mediated in vitro reconstitution of the 2Fe-2S cluster in apo-ferredoxin. During incubation with CpNifS in a reconstitution mix, CpIscA was shown to acquire a transient Fe-S cluster. The Fe-S cluster could subsequently be transferred by CpIscA to apo-ferredoxin. We propose that the CpIscA protein serves as a scaffold in chloroplast Fe-S cluster assembly.

Iron (Fe)-sulfur (S) clusters are cofactors of proteins that perform a number of biological roles, including electron transfer; redox and nonredox catalysis; regulation of gene expression; and as sensors for Fe and oxygen within all living organisms, prokaryotes, and eukaryotes (Beinert, 2000). Although Fe-S clusters can be assembled in proteins in vitro with ferrous Fe and sulfide, it is now clear that the process is not spontaneous in vivo, and proteins have been shown to be required for the biological formation of these clusters (for review, see Lill and Kispal, 2000; Frazzon et al., 2002). Genetic and biochemical studies in microorganisms initially led to identification of two types of Fe-S cluster machinery, termed NIF (nitrogen fixation) and ISC (iron-sulfur cluster; Zheng et al., 1993, 1998). A third machinery (SUF) with homologs in a wide range of organisms was characterized more recently (Takahashi and Tokumoto, 2002).

The proposed mechanism of cluster formation is as follows: (1) S is mobilized from Cys by the action of a Cys desulfurase enzyme (Zheng et al., 1993, 1998); (2) the S atoms are transferred to a scaffold protein (Urbina et al., 2001), Fe atoms are supplied to the scaffold protein, and the transient Fe-S cluster is assembled; and (3) the Fe-S cluster is inserted into various apo-proteins to form the Fe-S proteins (Yuvaniyama et al., 2000; Agar et al., 2000a; Krebs et al., 2001; Wu et al., 2002; Tong et al., 2003). In addition to Cys desulfurase and scaffold proteins, other factors such as Hsp70- and Hsp40-type chaperones as well as ferredoxin (Fd)/Fd reductase systems may be involved (Lill and Kispal, 2000; Frazzon et al., 2002). There are indications that molecular chaperone proteins interact with the scaffold protein and keep the scaffold protein in a conformation that facilitates the Fe-S cluster assembly and the transfer to an apo-Fe-S protein (Hoff et al., 2000; Silberg et al., 2001; Muhlenhoff et al., 2003).

In the nitrogen-fixing bacterium *Azotobacter vinelandii*, NifU was shown to provide a scaffold for Fe-S cluster formation (Zheng et al., 1993, 1994; Agar et al., 2000a; Yuvaniyama et al., 2000). In non-nitrogen-fixing bacteria and eukaryotic mitochondria, an IscU scaffold protein is essential and the main protein for Fe-S cluster biosynthesis; this protein shows a high sequence identity with the N-terminal domain of *A. vinelandii* NifU (Agar et al., 2000a, 2000b). IscU proteins accept S from a NifS-like Cys desulfurase and are also the binding site for Fe to build an Fe-S cluster (Agar et al., 2000b). By contrast, plastids and most non-nitrogen-fixing cyanobacteria whose genome sequence is known do not possess any homolog of IscU. Instead, proteins, termed NFUs, with sequence similarity to the C-terminal domain of *A. vinelandii* NifU were found to be scaffolds for Fe-S cluster assembly (Leon et al., 2003; Yabe et al., 2004). SyNifU, the NifU of cyanobacterium *Synechocystis*, serves as a scaffold for Fe-S cluster assembly and delivery (Nishio and Nakai, 2000).

IscA is another protein that may function as a scaffold protein for Fe-S cluster synthesis in *Escherichia coli*

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(Takahashi and Nakamura, 1999; Tokumoto and Takahashi, 2001), yeast (*Saccharomyces cerevisiae*; Jensen and Culotta, 2000; Kaut et al., 2000), and cyanobacteria (Morimoto et al., 2002; Wollenberg et al., 2003). It is proposed that the IscA family of proteins provide alternative scaffolds to the NifU and IscU proteins for mediating Nif-specific and general Fe-S cluster assembly (Krebs et al., 2001). In addition to a scaffold function in cluster formation in *E. coli*, it was proposed that IscA can provide Fe for the assembly of the transient Fe-S cluster in IscU in the presence of IscS and Cys in vitro (Ding and Clark, 2004). In *Synechocystis* PCC 6803, IscA1, the product of SLR1417, predominantly binds the Fe ion alone, whereas IscA2, the product of SLR1565, binds a 2Fe-2S cluster (Morimoto et al., 2002). IscA2 forms a complex with a HEAT-repeat-containing protein, IaiH, which stabilizes the Fe-S cluster in IscA2 (Morimoto et al., 2002).

In addition, the SUF gene clusters of *E. coli* and *Erwinia chrysanthemi* have genes that encode components similar to IscS and IscA, named SufS and SufA, respectively (Takahashi and Tokumoto, 2002). These proteins are thought to be involved in Fe-S cluster formation under Fe limitation and oxidative stress conditions (Ollagnier-de-Choudens et al., 2001; Outten et al., 2003, 2004).

In plant cells, mitochondria and chloroplasts are believed to originate from endosymbiotic bacteria and therefore are predicted to have their own Fe-S cluster biosynthesis machinery. The structural and physiological differences between mitochondria and chloroplasts suggest the existence of two distinct Fe-S assembly machineries. Mitochondria are a site of oxygen consumption, whereas chloroplasts produce oxygen through the photosynthetic process. Based on sequence homology of putative components, the mitochondrial Fe-S machinery may be most similar to the bacterial ISC machinery, where IscU is regarded as the major and essential scaffold protein for the cluster assembly. In chloroplasts, the components of the Fe-S machinery are starting to be identified. A NifS-like protein (AtCpNifS) with Cys desulfurase activity was localized in the chloroplast (Leon et al., 2002; Pilon-Smits et al., 2002). This protein is similar in sequence to a cyanobacterial L-Cys/L-cystine *C-S* lyase (C-DES; Lang and Kessler, 1999; Kessler, 2004). Other putative components include three NFU proteins (NFU1–3), similar to cyanobacterial NFU-like scaffold proteins and to the C terminus of *A. vinelandii* NifU (Leon et al., 2003; Yabe et al., 2004). Nfu2 purified from *E. coli* contained a transient Fe-S cluster (Leon et al., 2003) that could be passed on to apo-Fd in vitro (Yabe et al., 2004). Nfu2 insertion mutants have a dwarf phenotype and are deficient in some but not all plastid Fe-S proteins (Touraine et al., 2004; Yabe et al., 2004). Other potential Fe-S candidate proteins in plastids are the Arabidopsis homologs of SufBCD and E and the high chlorophyll fluorescence 101 protein (HCF101). The putative SufC homolog AtNAP7 is confirmed to be in the chloroplast, and mutants have phenotypes that indicate a role in plastidic Fe-S cluster maintenance and repair during Arabidopsis embryogenesis (Xu and Moller, 2004). HCF101 encodes a NifH-related P-loop ATPase that seems required for 4Fe-4S but not 2Fe-2S clusters in chloroplasts (Lezhneva et al., 2004). So far, a link of either NFUs or CpSufBCDE with CpNifS has not been made. Here, we report the cloning, purification, and characterization of a chloroplast-localized IscA-like protein (AtCpIscA) from Arabidopsis. CpIscA is shown to act as a molecular scaffold in Fe-S cluster biosynthesis in vitro and to be able to transfer the cluster to apo-Fd producing active holo-Fd.

RESULTS

Cloning of CpIscA and Sequence Analysis

In earlier studies, the NifS-like protein AtCpNifS from Arabidopsis was shown to have Cys desulfurase activity and to be located in the chloroplast (Leon et al., 2002; Pilon-Smits et al., 2002). To identify another Arabidopsis protein(s) that might be involved in Fe-S cluster formation in plastids, a database search was performed to identify homologs of the cyanobacterial *Synechocystis* PCC 6803 IscA proteins. BLAST searches in the Munich Information Center for Protein Sequences (http://mips.gsf.de/proj/thal/db/) and The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org) databases revealed the presence of one IscA-like protein (At1g10500). TargetP predictions suggest that this protein is a chloroplast protein with a 55-amino-acid-long chloroplast transit sequence. The corresponding gene was named AtCpIscA, and its cDNA was cloned using reverse transcription-PCR. Alignment of the AtCpIscA primary sequence with IscA- and SufA-like proteins from cyanobacteria and *E. coli* reveals the presence of three conserved Cys residues (residues 104, 170, and 172), including the characteristic motif CXC and a conserved Asp (residue 149) in the C-terminal region (Fig. 1A). These residues are conserved among all IscA-type proteins from prokaryotic and eukaryotic organisms. In addition to the three Cys conserved in all IscA-type proteins, AtCpIscA contains two other Cys, at residues 94 and 135, which are also present in cyanobacterial IscA1 from *Synechocystis* PCC 6803. AtCpIscA exhibits higher sequence similarity to IscA1 (Slr1417) from *Synechocystis* PCC 6803 (58% identity) and IscA from *E. coli* (47% identity) compared to the mitochondrial IscA-like protein from Arabidopsis (At2g16710; 24%) and SufA from *E. coli* (25%). Consequently, in a phylogenetic analysis, AtCpIscA was grouped with cyanobacterial IscA1 and *E. coli* IscA, separate from *E. coli* SufA and the Arabidopsis mitochondrial NifA-like protein (Fig. 1B).

Expression Analysis of CpIscA

The AtCpIscA gene expression pattern in different tissues was analyzed using RNA-blot analysis (Fig. 2).

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AtCpIscA       MAFATGITTSSNPTFLGLKISNTSLRSVVSCNSISFPSLSYVNLNLNRRNRLSVRSASVPAAPA 64
At2g16710      --------------------------------------------------MKASQILAAAAARVG 15
E.coli SufA    ------------------------------------------------------MDMHSGTFNP 10
E.coliIscA     ---------------------------------------------------------------- 0
Cyano-IscA1    ----------------------------------------------------MSQATATQAKG 11
Cyano-IscA2    ---------------------------------------------------------------- 0

AtCpIscA       MEGLKPAISLSENALKHLSKMRSERGED-LCLRIGVKQGGCSGMSYTMDFENRANARPDDSTIE 127
At2g16710      PALRKQVLTLTDEAASRVHHLLQ-QRQKPF-LRLGVKARGCNGLSYTLNYAD--EKGKFDELVE 75
E.coli SufA    QDFAWQGLTLTPAAAIHIRELVA-KQPGMIGVRLGVKQTGCAGFGYVLDSVS--EPDKDDLLFE 71
E.coliIscA     -----MSITLSDSAAARVNTFLA-NRGKGFGLRLGVRTSGCSGMAYVLEFVD--EPTPEDIVFE 56
Cyano-IscA1    -------IQLSDAALKHLLALKEQQGKD-LCLRVGVRQGGCSGMSYMMDFEEPNRATEHDEVFD 67
Cyano-IscA2    ------MLQLTPSAAQEIKRLQHSRQLTRHHFRLAVRPGGCAGWLYHLDFVP--EITADDLEYE 56
                       : *:   *    .:   :    .          .*:.*:  ** *   * ::       .     *   :

AtCpIscA       YQGFTIVCDPKSMLFLFGMQLDYSDALIGGGFSFSNPNATQTCGCGKSFAAEM---------   180
At2g16710      EKGVRILVEPKALMHVIGTKMDFVDDKLRSEFVFINPNSQGQCGCGESFMTTSTSSAKQSAS   137
E.coli SufA    HDGAKLFVPLQAMPFIDGTEVDFVREGLNQIFKFHNPKAQNECGCGESFGV-----------   122
E.coliIscA     DKGVKVVVDGKSLQFLDGTQLDFVKEGLNEGFKFTNPNVKDECGCGESFHV-----------   107
Cyano-IscA1    YEGFQIICDRKSLLYLYGLMLDYSNALIGGGFQFTNPNANQTCGCGKSFGV-----------   118
Cyano-IscA2    SGGVTVLVDSQSAGYLHNLKLDYAEDLMGGGFRFTNPNAAQVCSCSLSFAPNLEKNL-----   113
                 *   :.    ::   .: .   :*:        :    * * **:     *.*. **
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B

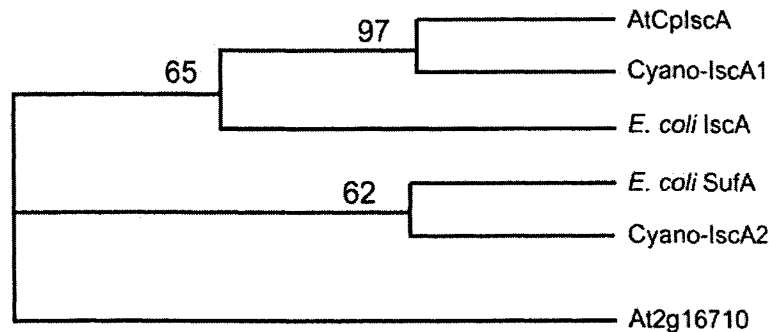

Figure 1. Sequence alignment and phylogenetic tree of IscA-like proteins from various organisms. A, Sequence alignment. The predicted chloroplast targeting sequence is underlined. Conserved amino acids are marked by asterisks, and the conserved Cys residues are boxed. The conserved Asp residue is shown in gray. Sequences are: AtCpIscA, At1g10500 (this article); At2g16710, encoding the putative Arabidopsis mitochondrial IscA-like protein; *E. coli* SufA (NC_004431); *E. coli* IscA (NC_002655.2); cyano-IscA1, *Synechocystis* IscA Slr1417; and cyano-IscA2, *Synechocystis* IscA Slr1565. B, Phylogenetic tree of IscA-like proteins used in the sequence alignment.

Ten micrograms of total RNA extracted from roots, stems, leaves, and flowers was electrophoresed, and ethidium bromide staining was used to verify equal loading. Northern-blot analysis reveals that AtCpIscA is expressed in all tested tissues, with higher expression level in green photosynthetic tissues (leaves and stems) than non-green tissues (roots and flowers; Fig. 2).

Intracellular Localization of CpIscA

The TargetP program (Emanuelsson et al., 2000) predicted a chloroplast localization of AtCpIscA and a cleavable transit sequence of 55 amino acids. To examine the subcellular localization, we constructed fusions with the green fluorescent protein (GFP). *GFP* was fused to the coding region for the predicted chloroplast transit sequence of CpIscA (TP-IscA-GFP) and to the full-length IscA including its transit sequence (Full length-IscA-GFP). *GFP* alone expressed from the same constitutive promoter was used as a control. The localization in cells was analyzed using confocal laser microscopy. Fluorescence corresponding to GFP expressed without a transit sequence was excluded from the chloroplasts as expected (Fig. 3, top). By contrast, green fluorescence from TP-IscA-GFP was localized to the chloroplast stroma, as indicated by the overlay of green fluorescence and red autofluorescence (Fig. 3, middle). Because chloroplast transit sequences effectively mediate translocation of a passenger protein across the envelope (Keegstra and Froehlich, 1999), this is the expected location for a transit sequence fusion. Interestingly, green fluorescence from the full-length CpIscA coupled to GFP (Full length-IscA-GFP) was localized to discrete locations in the chloroplast stroma, which may be indicative of the inclusion in discretely localized complexes (Fig. 3, bottom).

To investigate the chloroplast localization of CpIscA by an alternative method and to determine the size of the mature CpIscA protein in plastids, we performed

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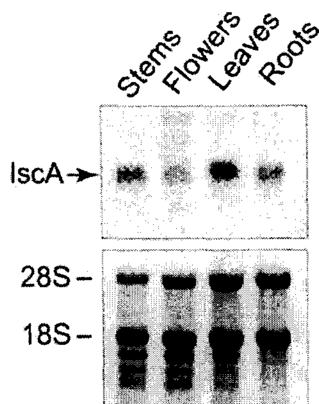

Figure 2. Expression analysis of CpIscA in different tissues. Total RNA from roots, leaves, stems, and flowers of 4-week-old Arabidopsis plants was isolated. Ten micrograms of total RNA was separated by electrophoresis, transferred to a Hybond-N membrane, and probed with ^{33}P-labeled CpIscA cDNA (top). Ethidium bromide-stained agarose gel was used to show loading (bottom). 28S and 18S are ribosomal RNA subunits.

an in vitro chloroplast uptake experiment (Fig. 4A). Radiolabeled precursor protein was produced by in vitro transcription of the cloned cDNA and subsequent translation of the synthetic mRNA in the presence of ^{35}S-Met. The translation reaction (Fig. 4A, right, PR) resulted in a radiolabeled protein band with a size expected for the precursor. In addition to the precursor, two translation products with a size smaller than the expected mature protein were present that may result from initiation at either of the two downstream AUG codons corresponding to residues 65 and 85. The translation mixture including the precursor was incubated with purified intact chloroplasts in the light and in the presence of ATP. After import and treatment with protease, the chloroplasts were recovered and proteins were analyzed by SDS-PAGE (Fig. 4A). The *E. coli*-expressed recombinant mature CpIscA was electrophoresed in the same gel to allow a direct comparison of the molecular mass with the imported protein. Chloroplasts incubated with the precursor encoded by CpIscA and treated with protease accumulated a protein of about 14 kD in size that has the same electrophoretic mobility as the purified recombinant mature protein. Quantitation of the precursor and the mature bands indicated that 32% of the added CpIscA precursor was imported in this assay, which was comparable to the plastocyanin control (data not shown). We conclude that the CpIscA precursor contains plastid-targeting information and that the protein is active in the chloroplast. The recombinant protein purified from *E. coli* corresponds in size to the imported mature CpIscA.

To analyze localization and expression levels in Arabidopsis plants, we raised an antibody (see "Materials and Methods") that was used in immunoblots to analyze the presence of CpIscA in total leaf homogenate and chloroplast stroma fractions. The antibody recognized a band corresponding to the size of mature CpIscA in total homogenate and isolated stroma, confirming the stromal localization of AtCpIscA (Fig. 4B). The mobility of the detected CpIscA protein band in

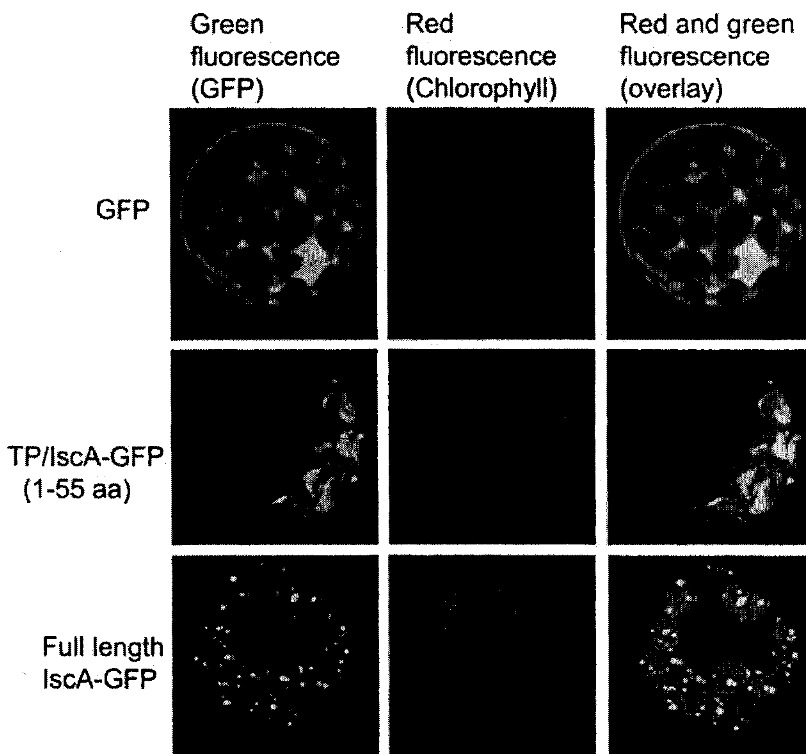

Figure 3. Subcellular localization of AtCpIscA. Arabidopsis protoplasts were transformed with plasmids that express the indicated gene constructs under control of the constitutive 35S cauliflower mosaic virus promoter. The nonfused GFP protein-coding sequence was used as a control. TP/IscA-GFP encodes the fusion of the CpIscA TP (1–55) to the N terminus of GFP, and full-length IscA GFP contains the full coding sequence of the IscA precursor fused to GFP. After 16 h of expression, cells were observed using a confocal laser-scanning microscope. Green fluorescence signals, chlorophyll red autofluorescence, and an overlay of green and red signals are shown.

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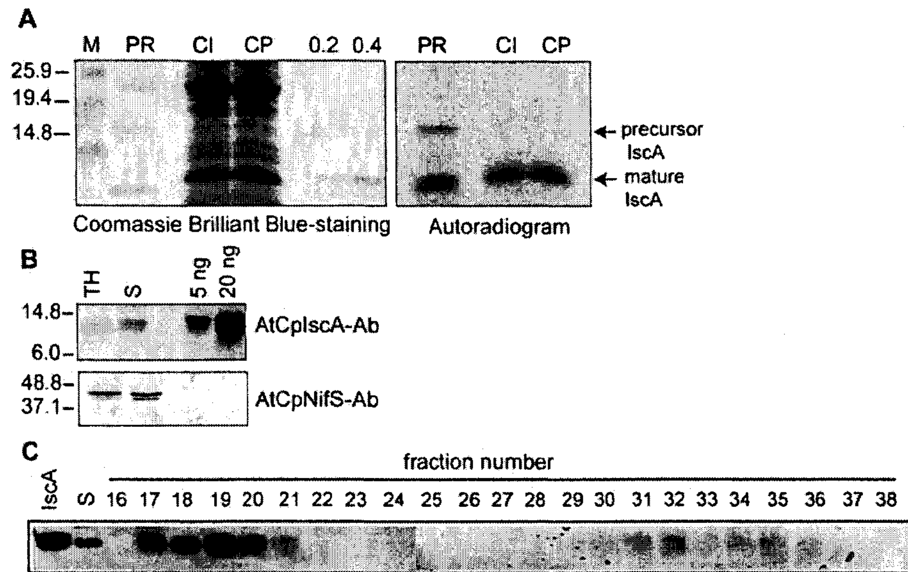

Figure 4. Chloroplast targeting of AtCpIscA and gel filtration analysis. A, In vitro import of radiolabeled CpIscA precursor. ^{35}S-Met-labeled Precursor CpIscA protein (PR) was obtained by in vitro translation and incubated with isolated pea chloroplasts. Chloroplasts were reisolated, and proteins in the intact chloroplasts were analyzed either directly (CI) or after treatment of intact chloroplast with protease (CP). Proteins were separated by 15% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue (left), and the radiolabeled protein bands were visualized using a PhosphorImager (right). B, Immunoblot analysis of 20 μg of total leaf homogenate protein (TH) and 20 μg of chloroplast stroma (S). Purified CpIscA (5 ng and 20 ng) was applied for comparison in the two right lanes of Figure 4B. AtCpNifS antibody (bottom) was used as a control for stromal protein. C, Gel filtration analysis of AtCpIscA in the stroma. One milligram of stromal protein was separated on a Superdex-S200 gel filtration column. Elution was monitored by the A_{280} (data not shown) and by immunoblotting of collected fractions with CpIscA-specific antibodies. The void volume of this column corresponds to fraction 11.

plant fractions is the same as that of purified recombinant CpIscA. Control experiments showed that these bands were not detected with preimmune serum (data not shown). Based on comparisons of the CpIscA staining intensities in stromal fractions and purified protein, we estimate the abundance of CpIscA in stroma to be approximately 0.01% to 0.02% of protein, which is slightly lower than the measured abundance of CpNifS (Ye et al., 2005). To investigate the oligomeric state of CpIscA in the stroma, a gel filtration experiment was performed using a high-resolution column and detection of AtCpIscA using immunoblotting (Fig. 4C). Approximately 90% of the stromal IscA was eluted in a single peak at high molecular mass (approximately 600 kD, fractions 17–20). In addition, a smaller amount of IscA was eluted as a dimer. This result indicates that CpIscA present in stroma interacts with other proteins in vivo and may form a transient complex with them. Interestingly, a fraction of CpNifS was also detected in a complex of approximately 600 kD (Ye et al., 2005), and coelution of CpIscA and CpNifS was confirmed by gel filtration (data not shown).

Expression and Purification of AtCpIscA

The coding sequence for mature CpIscA (without transit sequence) was cloned into the expression vector pET11d, and the mature-sized protein was expressed in BL21 codon$^+$ *E. coli* cells. Three hours after induction with isopropyl-β-D-thiogalactopyranoside (0.4 mM), a protein with a molecular mass of about 14 kD accumulated to about 5% to 10% of the soluble protein, as shown by Coomassie Brilliant Blue staining (Fig. 5, lane 1). This molecular mass is in agreement with the molecular mass calculated from the DNA sequence (13,971 D). Soluble IscA was purified using cation exchange chromatography and a gel filtration step (Fig. 5, lanes 2 and 3). The protein was eluted from the calibrated S200 gel filtration column with an apparent molecular mass of 54 kD, suggesting that the protein is purified as a tetramer (data not shown). N-terminal sequencing (Macromolecular Resources, Colorado State University) by Edmann degradation yielded the sequence RNRLSV, corresponding to the predicted N-terminal sequence of mature CpIscA. The pure CpIscA protein was colorless, indicating it did not contain an Fe-S cluster while in *E. coli.*

CpIscA with CpNifS Stimulates Fe-S Cluster Formation in Fd

To study the role of CpIscA in Fe-S cluster formation, an in vitro reconstitution assay was developed. In this assay, apo-Fd was reconstituted to the holo-form by acquiring an Fe-S cluster, which was synthesized in vitro from Cys S and a ferrous Fe salt. Holo-Fd was

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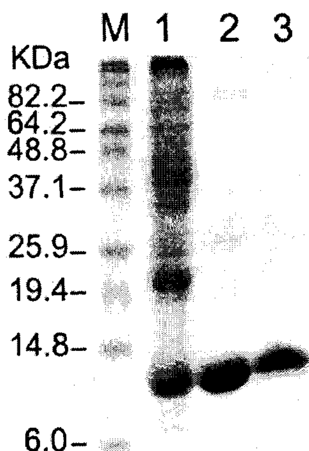

Figure 5. SDS-PAGE of IscA at different stages of its purification. Lane 1, Molecular mass marker; lane 2, total lysate from *E. coli* BL21 (DE3)/codon+ expressing IscA; lane 3, IscA after cation exchange column; lane 4, IscA after HPLC gel filtration column.

separated from apo-Fd and other proteins and quantified by HPLC using an ion-exchange column. The activity of CpNifS proved to be sufficient and required for the Fe-S cluster formation in Fd (Ye et al., 2005). However, other proteins may promote the efficiency of CpNifS-mediated holo-Fd formation in a plant cell. In the presence of all necessary substrates for Fe-S cluster formation, except CpNifS, CpIscA alone did not show any reconstitution activity. However, preincubation of CpIscA with CpNifS significantly increased subsequent Fd reconstitution activity compared to that shown by CpNifS alone ($P < 0.05$; Fig. 6). The observed modest increase in reconstitution activity was a first indication of a possible role of CpIscA in Fe-S cluster formation.

Incorporation of an Fe-S Cluster into CpIscA

The stimulation of CpNifS-dependent Fd reconstitution activity by CpIscA suggested possible interactions between CpIscA and CpNifS in the Fe-S cluster formation where CpIscA may be a scaffold for cluster assembly and an intermediate in Fe-S insertion. To investigate this possibility in more detail, CpIscA (300 μg) was incubated with CpNifS (75 μg) in reconstitution buffer (5 mM dithiothreitol [DTT], 1 mM L-Cys, 1 mM ferrous ammonium sulfate, 20 μM pyridoxal 5′ phosphate [PLP]) for 30 min and then applied to a gel filtration column (Fig. 7A, line 3). As controls, apo-CpIscA (300 μg) incubated in gel filtration buffer or in reconstitution buffer, as well as CpNifS alone (75 μg), were incubated and run through the same column (Fig. 7A, lines 1, 2, and 4, respectively). Proteins were detected by A_{280} (Fig. 7) and 420 nm (data not shown). Purified untreated apo-CpIscA eluted from the column in a peak with a retention time expected for the tetramer but a shoulder was present, suggesting the existence of lower-molecular mass IscA species (Fig. 7A, line 1). Incubation of ApoIscA with DTT-containing buffer resulted in mainly the dimeric form of the protein (line 2). No absorbance was detected at 420 nm, indicating the absence of Fe-S clusters in the apo-IscA fractions (data not shown). As shown in Figure 7A (line 3), incubation of CpIscA with CpNifS resulted in two major peaks corresponding to CpNifS and a CpIscA dimer, according to its calculated molecular mass. SDS-PAGE (Fig. 7B) indicated that the peak of CpNifS eluted at 17.5 min (fraction 28) and the dimeric CpIscA at 20.5 min (fraction 34). Interestingly, the peak corresponding to reconstituted dimeric CpIscA could also be detected by A_{420} (data not shown), suggesting the presence of an Fe-S cluster. Apo-CpIscA (line 2) and reconstituted dimeric CpIscA (line 3) were collected from gel filtration runs, and their absorption spectra were measured (Fig. 7C). Unreconstituted CpIscA showed only one peak at 280 nm, characteristic for aromatic residues in proteins. Reconstituted CpIscA (fraction 34), however, showed extra maxima at 330 nm, 420 nm (with a shoulder at 470), and 580 nm, indicative of a 2Fe-2S cluster (Fig. 7C). In a typical 2Fe-2S cluster, absorption from 420 nm to 460 nm is attributed to the vibration between Fe and bridging inorganic S (Morimoto et al., 2002).

The presence of Fe in CpIscA was determined chemically (see "Materials and Methods") using spinach (*Spinacia oleracea*) holo-Fd as control, which contains one 2Fe-2S cluster per protein. We measured 2.0 ± 0.2 Fe/protein for spinach holo-Fd. Reconstituted CpIscA contained 1.4 ± 0.2 Fe/dimer, while no Fe was detected in the apo-form. Because the reconsti-

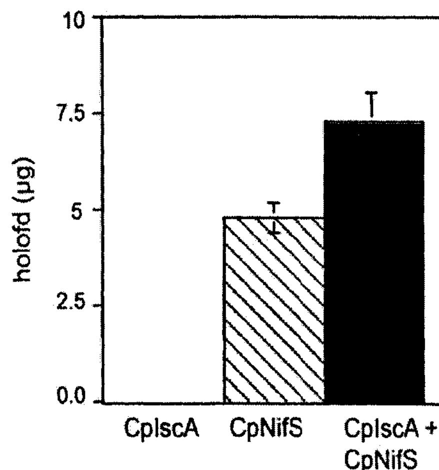

Figure 6. Purified CpIscA enhances the Fd reconstitution activity of CpNifS. Purified CpNifS (7.5 μg), CpIscA (30 μg), or a combination of both CpNifS (7.5 μg) and CpIscA (30 μg) were incubated with 50 mM Tricine-NaOH, pH 7.5, 5 mM DTT, 1 mM L-Cys, 1 mM ferrous ammonium sulfate, 20 μM PLP for 30 min, followed by addition of 30 μg of apo-Fd. After 20 min, the reconstituted holo-Fd was assayed by HPLC. Data represent the average and SE of three replicates.

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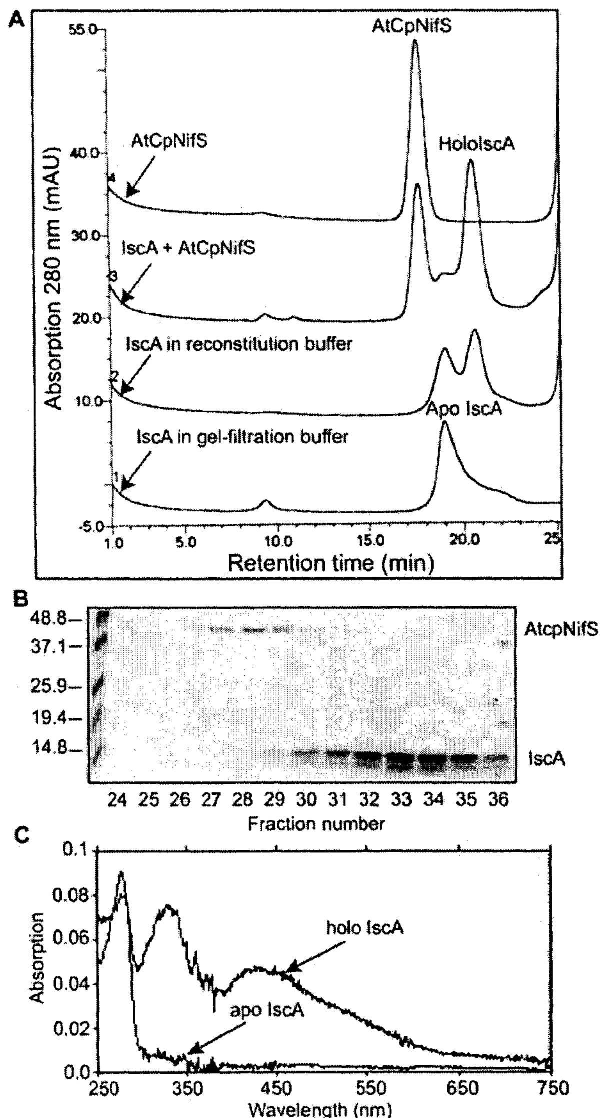

Figure 7. Purified CpIscA can acquire an Fe-S cluster. A, Gel filtration analysis. CpIscA (300 μg) was incubated with CpNifS (75 μg) in reconstitution buffer (50 mM Tricine-NaOH, pH 7.5, 5 mM DTT, 1 mM L-Cys, 1 mM ferrous ammonium sulfate, 20 μM PLP; line 3) for 30 min. CpIscA (300 μg) in gel filtration buffer (25 mM Tricine/KOH, pH 7.9, 50 mM KCl; line 1) or in reconstitution buffer (reaction 2) and CpNifS (75 μg) in reconstitution buffer (reaction 4) were used as controls. After incubation at 37°C, proteins were separated on a Superdex S200 gel filtration column equilibrated in 25 mM Tricine/KOH, pH 7.9, 50 mM KCl, and elution was monitored by the A_{280}. NifS eluted at 17.5 min (fraction 28), apo-IscA (tetramer) at 19 min (fraction 31), and holo-IscA (dimer) at 20.5 min (fraction 34). B, SDS-PAGE of gel filtration fractions of treatments shown in line 3. C, Absorption spectrum of apo-CpIscA and holo-CpIscA. The protein concentration was 300 μg in 25 mM Tricine/KOH, pH 7.9.

tuted fraction obtained after incubation with CpNifS (line 3) still may contain some inactive apo-CpIscA, a number slightly lower than two is expected for the number of Fe atoms/dimer. These data indicate that the isolated and reduced CpIscA is a clusterless apo-protein, while the reconstituted dimer may contain a 2Fe-2S cluster. The Fe-S cluster formed in IscA is stable since treatment with Fe chelator (1 mM EDTA) or overnight incubation at 4°C did not induce destruction of the Fe-S cluster, as indicated by the reconstitution efficiency (data not shown).

Fe-S Cluster Transfer from CpIscA to Apo-Fd

As CpIscA appeared to have an Fe-S cluster, the potential transfer of the Fe-S cluster to apo-Fd was investigated. Both apo- and holo-CpIscA (150 μg) were collected from the gel filtration column and incubated with 30 μg of apo-Fd in the presence of 5 mM DTT at 37°C for 30 min. As controls, holo-Fd (30 μg) alone and holo-IscA (150 μg) alone were incubated. Incubation mixtures were used for holo-Fd analysis by HPLC. As shown in Figure 8A, the apo-CpIscA fraction did not lead to any formation of holo-Fd, while 50% (15 μg) of apo-Fd was reconstituted to holo-Fd by acquiring an Fe-S cluster from the dimeric holo-CpIscA. To test if the amount of holo-Fd formation depends on the amount of holo-IscA, various amounts of holo-IscA were incubated with apo-Fd (30 μg) for 30 min and the amount of holo-Fd was assayed (Fig. 8B). The amount of holo-Fd formed increased with increasing the amount of holo-IscA. Importantly, in the same condition when holo-IscA (60 μg) was incubated with apo-Fd (30 μg) in the presence of 1 mM EDTA, reconstitution was still observed with 70% activity of the reaction without EDTA indicating direct transfer from IscA to Apo-Fd (data not shown). Thus, it is proposed that holo-CpIscA can be formed from Apo-CpIscA by acquisition of a transient Fe-S cluster, which it can subsequently deliver to apo-Fd. This suggests a role for CpIscA as a scaffold during Fe-S cluster formation in plastids and in transfer of the formed cluster to apo-Fe-S proteins.

DISCUSSION

Recently, it was demonstrated that AtCpNifS, a chloroplastic NifS-like Cys desulfurase of Arabidopsis, is responsible for the release of S from Cys for the biogenesis of Fe-S clusters in vitro (Ye et al., 2005). In this study, we report that Arabidopsis chloroplasts contain an IscA-like protein, CpIscA, which enhances the CpNifS-dependent Fe-S cluster formation in vitro. In addition, we provide evidence that recombinant apo-CpIscA, upon incubation with AtCpNifS and appropriate substrates, can acquire an Fe-S cluster, which in turn can be donated to apo-Fd in vitro. From these observations, we hypothesize that CpIscA functions as a scaffold protein for the assembly of transient Fe-S clusters from Fe and elemental S, which can be donated to Fe-S apo-proteins in the plastids. These results are of significance since Fe-S cluster proteins play crucial roles in essential plant processes, such as photosynthesis, which determine plant productivity

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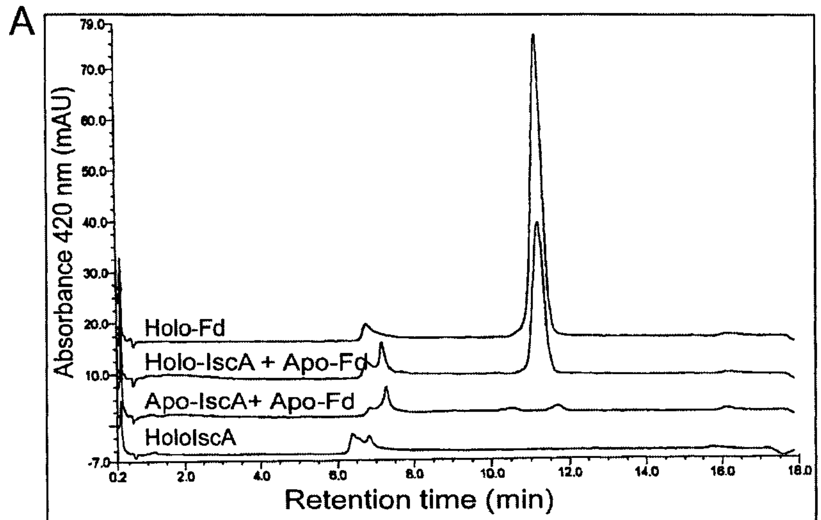

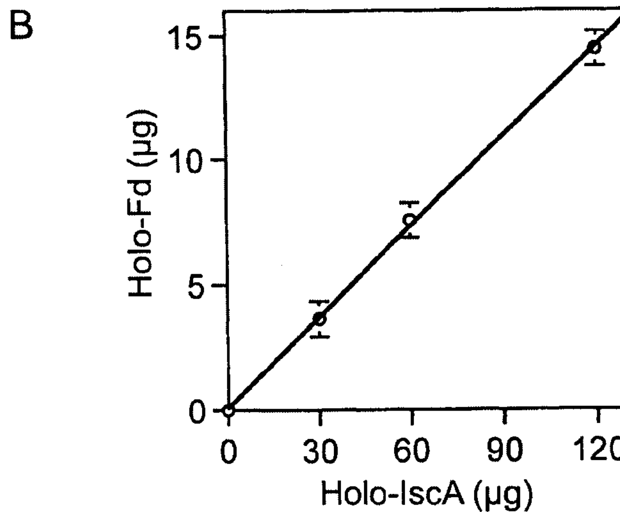

Figure 8. Fe-S cluster transfer from CpIscA to Fd apo-protein. A, Fe-S cluster transfer from holo-IscA to apo-Fd. Apo-Fd (30 μg) was incubated with 150 μg of holo-CpIscA (collected from gel filtration column, reaction 3, Fig. 7) or 150 μg of apo-CpIscA purified by gel filtration column. After 30 min, the Fd was assayed by HPLC and quantified. Holo-Fd (30 μg) and holo-IscA (150 μg) were used as controls. B, The amount of holo-Fd formed depends on the amount of holo-IscA. Thirty micrograms of apo-Fd was incubated with various amounts of holo-IscA for 30 min, and the amounts of holo-IscA were assayed by HPLC and quantified as before.

and nutritional value. Although plastids are known to be able to synthesize their own Fe-S clusters (Takahashi et al., 1986), the machinery involved in Fe-S cluster formation in plastids remains largely to be elucidated. The results presented here contribute to the understanding of this important process.

CpIscA has three conserved Cys residues and a conserved Asp residue (Fig. 1) with the sequence arrangement $C\text{-}X_{42\text{-}44}\text{-}D\text{-}X_{20}\text{-}C\text{-}G\text{-}C$. These residues are conserved among all IscA-type proteins from prokaryotic and eukaryotic organisms, including *nif*-specific IscA proteins (Krebs et al., 2001). This sequence motif of IscA scaffold proteins is different from the primary sequence arrangement of Cys in NifU/IscU scaffold proteins ($C\text{-}G\text{-}D\text{-}X_{22\text{-}24}\text{-}C\text{-}X_{43}\text{-}C$; Krebs et al., 2001). Individual amino acid substitutions for each of the three conserved Cys residues of the yeast Isa1p and Isa2p proteins (IscA homologs) yield the same phenotype as the gene knockout (Jensen and Culotta, 2000). When any one of three conserved Cys residues in *Synechocystis* IscA2 was replaced with Ser, the amount of assembled 2Fe-2S was significantly reduced (Morimoto et al., 2003). It has been suggested that two of the three conserved Cys residues are involved in cluster binding, whereas the third one provides an electron during cluster assembly for the reduction of the Cys persulfide at NifS/IscS (Krebs et al., 2001). Further studies are required to investigate the role of these Cys residues in CpIscA.

Two scaffold proteins for Fe-S cluster formation in nitrogenase, called NifU and ^{nif}IscA, were discovered in the Nif operon of *A. vinelandii*. NifU consists of three domains: an N-terminal region that can accept a transient cluster, a central region with stable cluster, and a C-terminal thioredoxin-like domain known as the Nfu region (Agar et al., 2000a; Yuvaniyama et al., 2000). Homologs of the N-terminal domain of NifU as well as of NifA seem to function in the general Fe-S

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cluster machinery found in mitochondria (Lill and Kispal, 2000) and are also encoded by the Isc-gene cluster of bacteria such as *E. coli* (Frazzon et al., 2002). This Isc-type machinery was reported to be sensitive to oxygen in *E. coli*, *A. vinelandii*, and *Schizosaccharomyces pombe* (Krebs et al., 2001; Ollagnier-de-Choudens et al., 2001; Wu et al., 2002). In *E. coli*, a second Fe-S assembly machinery is encoded by the SUF operon and the gene cluster includes SufA, a homolog of IscA/NifA. The Suf operon may function in Fe-S assembly under conditions of oxidative stress and Fe limitation (Outten et al., 2004).

The CpIscA-mediated formation of the [2Fe-2S] cluster in apo-Fd proceeds under normal atmospheric (oxygenic) conditions in the presence of a thiol reductant (DTT) in the assembly cocktail. By comparison, the reconstituted Fe-S cluster in either *E. coli* or *A. vinelandii* IscA proteins was fairly labile (Ollagnier-de-Choudens et al., 2001; Krebs et al., 2001), and the cluster formed in CpIscA was stable and insensitive to oxygen, as indicated by the persistence over several days at 4°C. Since photosynthetic activity produces oxygen, this reaction condition may be appropriate for a chloroplast enzyme. By contrast, the mitochondrial Isc-type Fe-S protein maturation machinery requires the exclusion of oxygen in vitro (Muhlenhoff et al., 2002). Chloroplasts are proposed to be derived from an ancestral cyanobacterium-like endosymbiont (Martin et al., 1998). No IscU-type scaffold proteins have been identified in the nine completely sequenced genomes of cyanobacteria (http://www.ncbi.nlm.nih.gov/blast/Blast/.cgi). However, IscA scaffold proteins were identified in *Synechocystis* PCC 6803 (Wollenberg et al., 2003). Genes encoding IscU homologs with putative chloroplast-targeting sequences were not found in the Arabidopsis genome (TAIR; www.arabidopsis.org). This suggests that the Arabidopsis chloroplast, like cyanobacteria, does not express an IscU scaffold homolog. Therefore, the CpIscA protein may provide an alternative scaffold to the IscU proteins for Fe-S cluster formation and delivery in chloroplasts.

In addition to CpIscA, three chloroplast-localized CpNfu proteins were identified that may function as molecular scaffolds for Fe-S cluster biosynthesis (Leon et al., 2003; Touraine et al., 2004; Yabe et al., 2004). An interesting question that remains to be answered is whether the CpIscA and CpNfu scaffold proteins function complementary, overlapping, or parallel to each other in chloroplasts. Some plausible reasons for the need of alternative scaffolds are that each scaffold protein is optimized for assembly of either [2Fe-2S] or [4Fe-4S] clusters in vivo, that they preferentially transfer clusters to different acceptor proteins, or that each functions optimally under different physiological conditions, e.g. conditions that are more reducing or oxidizing. Complementary or partially overlapping roles in the delivery of Fe-S clusters to various substrate apo-proteins in different plastids and/or under different growth conditions were postulated for AtCpNfus (Touraine et al., 2004; Yabe et al., 2004). A scaffold role for CpIscA in plastid Fe-S cluster formation is similar to the finding that IscA from *E. coli* (Ollagnier-de-Choudens et al., 2001) and cyanobacterium *Synechocystis* PCC 6803 (Wollenberg et al., 2003) can serve as a scaffold for formation of a [2Fe-2S] cluster, which can be donated to the Fe-S apo-protein Fd. The homolog from *A. vinelandii* ^{Nif}IscA can also serve as a scaffold for 4Fe-4S clusters (Krebs et al., 2001). Once a suitable in vitro plant system for reconstitution of a 4Fe-4S apo-protein has been established, it will be interesting to investigate whether CpIscA serves as a scaffold for both types of Fe-S cluster.

MATERIALS AND METHODS

Cloning and Plasmid Construction

The Arabidopsis (*Arabidopsis thaliana*) CpIscA coding sequence was amplified by PCR using cDNA as a template. cDNA was prepared from DNase-treated total RNA prepared from 2-week-old seedlings as described (Pilon-Smits et al., 2002). Primers used for IscA amplification were 5′-GCTCTAGACAGAAGATTATGGCTTTCGC-3′ (forward primer) and 5′-TCCCCCGGGTGTGAAACTGGTTCACATCT-3′ (reverse primer). Underlined bases indicate *Xba*I and *Sma*I sites, respectively. The PCR product was digested with *Xba*I and *Sma*I and then ligated into a pBS (KS$^+$) vector (Stratagene, La Jolla, CA), digested with the same restriction enzymes to produce plasmid pPrIscA. To subclone the mature sequence of IscA in pET11d for expression, PCR was performed with another set of nested primers, 5′-CATGCCATGGCTGTTCGATCCGCTTCGGTTC-3′ (forward primer) and 5′-CGGGATCCTCACATCTCGGCAGCAAAAGA-3′ (reverse primer). Underlined bases indicate *Nco*I and *Bam*HI sites, respectively. The PCR product was digested with *Nco*I and *Bam*HI and subcloned in pET11d to produce plasmid pMIscA.

Construction of the plasmid for expression of the transit peptide (TP; amino acids 1–55) of IscA fused to GFP was performed as follows. The sequence encoding the predicted TP of IscA was amplified by PCR using flanking primers *Sal*I-N (5′-GAATGGTCGACATGGGCTTTCGCTACTGGAATCACG-3′) and *Nco*I-C-TP (5′-CATGCCATGGACATCTCGGCAGCAAAAGACTTCC-3′). To make a fusion of GFP with the full-length protein, the entire coding sequence of IscA was amplified by PCR using flanking primers *Sal*I-N and *Nco*I-C-Fl (5′-CATGCCATGGCTGATCTCATCTTACTCAGATGCTTAA-3′). pPrIscA plasmid was used as a template for PCR. PCR products were digested and inserted into the *Sal*I/*Nco*I-digested GFP reporter plasmid, 35Ω-SGFP(S65T), to create the TP/IscA-GFP and Full length-IscA-GFP, respectively. The inserts in all plasmids were sequenced by the dideoxy dye termination method to ensure that no error was introduced during PCR reactions. The plasmids used for protoplast transformation were prepared using the Plasmid Midi kit (QIAGEN, Valencia, CA).

Sequence Analysis and Alignments

Sequence analysis was performed using the Mac Vector sequence analysis software (International Biotechnologies, New Haven, CT). Searches for sequence similarity were performed using the BLAST network service provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Sequence alignment was performed using ClustalW at European Bioinformatics Institute, ExPASY Proteomics tools (http://www.ebi.ac.uk/clustalw). Phylogenetic analysis was performed using the PAUP (Sinauer Associates, Sunderland, MA; version 4.0b10) heuristic search method with tree bisection-reconnection branch swapping (Swofford, 1993). Bootstrap analysis with 1,000 random replicates was performed using the heuristic method.

In Vitro Chloroplast Import Assay

The pPrIscA plasmid was linearized with *Kpn*I and transcribed in vitro using T7 polymerase (Epicenter Technologies, Madison, WI) according to the manufacturer's instructions. Radiolabeled precursors were synthesized in

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a wheat germ lysate system in the presence of ^{35}S-Met (25 μCi/50 μL reaction; Amersham/Pharmacia, Piscataway, NJ) according to suggested protocols (Promega, Madison, WI). Chloroplasts for import experiments were isolated from 10-d-old pea (*Pisum sativum*) seedlings (cv Little Marvel) and incubated with radiolabeled precursor as described (Pilon et al., 1992). The postimport thermolysin treatment and reisolation of intact chloroplasts were performed as described (Smeekens et al., 1986). Proteins from import experiments equivalent to 10 μg of chlorophyll were separated by 15% SDS-PAGE, stained with Coomassie Brilliant Blue, fixed in 7% (v/v) acetic acid, 25% (v/v) methanol, dried, and the radiolabeled proteins visualized and quantified using a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Immunoblotting, Gel Filtration, and Antibody Production

Total homogenate and intact chloroplasts were isolated from rosette leaves of Arabidopsis plants as described by Rensink et al. (1989). A stromal protein fraction was obtained from chloroplasts as described by Smeekens et al. (1986). Gel filtration analysis was performed as described (Ye et al., 2005). Protein samples were separated by SDS-PAGE, transferred to cellulose membrane, and probed with CpIscA-specific antibody as described by Pilon-Smits et al. (2002). Polyclonal antibodies against bacterially expressed CpIscA were raised in rabbits at a commercial facility (Pocono Rabbit Farm and Laboratory, Canadensis, PA). Two rabbits were immunized after collection of preimmune sera. Preimmune serum at dilutions between 1/500 and 1/2,000 in phosphate-buffered saline did not recognize either purified CpIscA or CpIscA in plant homogenates. Antiserum from both rabbits detected CpIscA, and the optimal antiserum dilutions in phosphate-buffered saline were found to be 1/2,000 for immunoblots. The antibody for CpNifS has been described (Pilon-Smits et al., 2002).

RNA-Blot Analysis

Total RNA from different tissues (roots, leaves, stems, and flowers) was isolated by the TRIzol method (Invitrogen, Carlsbad, CA). Ten micrograms of total RNA was electrophoresed on a 1% (w/v) agarose gel containing 4% (w/v) formaldehyde, transferred to a nylon membrane, and probed with a ^{33}P-labeled *AtCpIscA* cDNA synthesized with a random primer labeling kit from Amersham. Prehybridization, hybridization, washing, and detection were done as described previously (Pilon-Smits et al., 2002).

Subcellular Localization of GFP-Fusion Proteins

GFP fusions were expressed in Arabidopsis protoplast-derived cells from the 35S promoter of cauliflower mosaic virus in the GFP reporter plasmid 35Ω-SGFP(S65T). For protoplast preparation, Arabidopsis plants (ecotype Columbia) were grown on Murashige and Skoog medium (Murashige and Skoog, 1962) for 3 weeks. Two grams fresh weight of leaf tissue was placed in 30 mL of a buffer containing 1% (w/v) cellulase Onozuka R-10, 0.25% Macerozyme R-10 (Karlan Research Products, Santa Rosa, CA), 8 mM $CaCl_2$, 0.5 M mannitol, 5 mM MES, pH 5.5, vacuum infiltrated for 1 min, and incubated for 3 h at room temperature with gentle shaking. The clear digest was filtered through a 37- to 70-μm nylon mesh (Carolina Biological Supply, Burlington, NC), and the protoplasts were harvested by centrifugation for 5 min at 1,000 rpm and washed twice in 10 mL of cold W5 wash solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM Glc, 0.5 M mannitol, adjusted to pH 5.8 with KOH). The pellet was suspended in about 2 mL of mannitol/magnesium solution (15 mM $MgCl_2$, 0.4 M mannitol, 0.1% MES, pH 5.6). Protoplasts were counted using a hemocytometer, and their concentration was adjusted to 3×10^6 cells mL^{-1} with mannitol/magnesium solution. Fifty micrograms of plasmid DNA, 100 μg of salmon sperm DNA, and 300 μL of polyethylene glycol (PEG) solution [40% PEG 6000/0.4 M mannitol/0.1 M $Ca(NO_3)_2$, adjusted to pH 8.0 with KOH] were added to 300 μL of the protoplast solution, very gently mixed, and left for 30 min at room temperature. The solution was very slowly diluted with 10 mL of W5 solution and then pelleted by centrifugation for 5 min at 1,000 rpm. Protoplasts were further resuspended in 5 mL of protoplast culture medium composed of Murashige and Skoog medium supplemented with 0.4 M Glc, 0.4 M mannitol, 1 mg/L 2,4-dichlorophenoxyacetic acid, 0.15 mg/L kinetin, pH adjusted to 5.8 with KOH, and left at 23°C for 16 h under continuous light. Confocal images were obtained using an Olympus Fluoview 300 inverted confocal laser-scanning microscope (1X70) equipped with an argon ion laser system and a 1.4 numerical aperture 60× objective lens (Olympus America, Melville, NY). The fluorescence signals were detected at 530 nm for GFP and 660 nm for chlorophyll. Sections of 1-μm thickness were scanned, and images were captured by the Fluoview software as Tiff files.

Preparation of Proteins

Fd was extracted from fresh spinach (*Spinacia oleracea*) that was obtained at a local health-food store, essentially as described (Yocum, 1982), and further purified as described (Pilon et al., 1992). The apo-Fd for reconstitution assays was prepared from holo-Fd by removal of the Fe-S cluster as described (Kato et al., 2000). Apo-Fd (at 0.5 mg/mL) was stored in 100 mM Tris-HCl, pH 8.0, under nitrogen in small aliquots at −80°C. CpNifS was expressed and purified as described (Pilon-Smits et al., 2002). For overexpression of AtCpIscA, *Escherichia coli* BL21 (DE3) codon$^+$ (Stratagene) was transformed with pMIscA vector, and 2 L of LB medium containing 50 μg mL^{-1} ampicillin was inoculated with 1/100 volume of overnight culture. Cells were grown at 37°C to an OD_{600} of 0.5, and expression was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C. The bacterial pellet was washed with 150 mM NaCl and resuspended in buffer A (25 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA) and passed twice through a French press (8,000 psi) at 4°C to disrupt the cells. All further manipulations were at 4°C unless indicated otherwise. The lysate was centrifuged for 20 min at 12,500*g*, and the cleared supernatant was loaded onto a cation-exchange SP Sepharose 1.6- × 20-cm column (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in 25 mM potassium phosphate, pH 7.5, 1 mM EDTA at a flow rate of 5 mL min^{-1}. The column was washed with three volumes of the same buffer and eluted with a 500-mL linear gradient of 0 to 0.5 M KCl in the same buffer, and 6-mL fractions were collected. Peak fractions were concentrated by adding ammonium sulfate to 70%, and the precipitated protein was collected by centrifugation for 20 min at 12,500*g*, dissolved in 5 mL of buffer A, and dialyzed overnight in 3 L of the same buffer. The dialyzed protein was filtered through a 0.2-μm filter and applied to a Sephacryl-S200 Hiprep 16/60 column (Amersham Bioscience, Piscataway, NJ) equilibrated in 25 mM potassium phosphate, pH 7.5, 100 mM KCl, 1 mM EDTA at room temperature and connected to a Summit HPLC system (Dionex, Sunnyvale, CA). The column was eluted, and fractions of 1 mL were collected. Elution was monitored by detection of the OD at 280 nm. The purified protein was concentrated as before and dissolved in the same buffer, without EDTA, dialyzed, aliquoted, and stored at −80°C with 15% (w/v) glycerol. Typical yields were 5 to 10 mg L^{-1} of culture. The N-terminal sequence was determined by Edmann degradation at the Colorado State University Macromolecular Resources facility.

Enhancement of Fe-S Cluster Assembly in Fd by IscA

The role of IscA in Fe-S cluster assembly was assayed by incubating IscA (30 μg) with NifS (7.5 μg) for 30 min in reconstitution buffer containing 50 mM Tricine-NaOH, pH 7.5, 5 mM DTT (Roche Diagnostics, Palo Alto, CA), 1 mM L-Cys (Sigma, St. Louis), 1 mM ferrous ammonium sulfate, and 20 μM PLP. Apo-Fd (30 μg) was added and incubated for 20 min at 37°C. After incubation, the reaction mixture was centrifuged at 14,000*g* for 1 min and directly applied to a 1-mL RESOURCE Q anion-exchange column (Amersham) connected to a summit HPLC system with a UVD170 detector and controlled by Chromeleon software (Dionex). The sample loop size was 100 μL. The column had been equilibrated with 25 mM Tris-HCl, pH 7.5. The following KCl gradient was applied in this buffer at a flow rate of 1.5 mL min^{-1}: 0 to 4.5 min, 0 M KCl; 4.5 to 5 min, 0 to 0.25 M KCl; 5 to 14 min, 0.25 to 0.55 M KCl; ramp up to 1 M KCl; 14.1 to 15.6 min, 1 M KCl; ramp down to 0 M KCl; and hold for 2 min to reequilibrate. Holo-Fd eluted at 10.8 min. Elution was monitored by absorbance at both 280 and 420 nm. Holo-Fd was quantified by signal integration at 420 nm, a characteristic absorption maximum for Fd. Spinach holo-Fd (30 μg in 150 μL) was used as a standard. The activity of the holo-Fd recovered from CpNifS/CpIscA reconstitution was assayed by examining NADP reduction (Smillie and Entsch, 1971). The Δ_{A340} was 0.27 mg^{-1} Fd min^{-1}, and this activity was light dependent. The absorption spectrum change was the same for both reconstituted Fd and spinach holo-Fd.

Incorporation of a Transient Fe-S Cluster into CpIscA, Gel Filtration Analysis, and Cluster Transfer

Incorporation of the Fe-S cluster into CpIscA was achieved by incubating IscA (300 μg) with 75 μg of NifS in reconstitution buffer (reaction 3; Fig. 7).

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Separate incubations of CpIscA (300 μg) in gel filtration buffer (25 mM Tricine/KOH, pH 7.9, 50 mM KCl; reaction 1; Fig. 7), IscA (300 μg) in reconstitution buffer (reaction 2), and CpNifS (75 μg) in reconstitution buffer (reaction 4; Fig. 7) were used as controls. After incubation at 37°C for 30 min, the mixtures were centrifuged at 14,000*g* for 1 min and directly applied to a Superdex-200 gel filtration column (1 × 30 cm; Pharmacia) connected to a summit HPLC system with a UVD170 detector and controlled by Chromeleon software (Dionex). The column was equilibrated in 25 mM Tricine/KOH, pH 7.9, 50 mM KCl. The flow rate was 0.75 mL min^{-1}, and fractions were collected every 0.5 min. A loop size of 500 μL was used. Elution was monitored by A_{280} and 420 nm. The void volume was determined with blue dextran. Standards used for calibration were IgY, bovine serum albumin, ovalbumin, chymotrypsinogen, and RNase.

Apo-Fd (30 μg) was incubated with holo-IscA, which was collected from a gel filtration column (reaction 3), for 30 min at 37°C in gel filtration buffer with 5 mM DTT. The resulting holo-Fd was assayed by HPLC as described (Ye et al., 2005). Gel filtration analysis of CpIscA in stromal fractions utilized the same column, buffer, and elution conditions as described above.

UV-Visible Spectroscopy

UV-visible absorption spectra (250–750 nm) were recorded with a Beckman DU 530 spectrophotometer (Beckman Instruments, Fullerton, CA) in a 100-μL quartz cuvette containing 20 to 30 μg of IscA protein in assay buffer (25 mM Tricine/KOH, pH 7.9, 50 mM KCl) in 1.0-nm scan steps.

General Methods

Protein was assayed according to Bradford using bovine serum albumin as a standard (Bradford, 1976). For CpIscA, the protein assay by Bradford was calibrated by measuring the protein absorption at 280 nm in 6 M guanidine HCl and calculation of absolute amounts using the molar extinction coefficient predicted by the primary sequence (Gill and von Hippel, 1989). Fe content of proteins was determined according to Fish (1988). Spinach holo-Fd was used as a control. Statistical analysis was performed with the JMP-IN software (SAS Institute, Cary, NC).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY971959.

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LITERATURE CITED

Agar JN, Yuvaniyama P, Jack RF, Cash VL, Smith AD, Dean DR, Johnson MK (2000a) Modular organization and identification of a mononuclear iron-binding site within the NifU protein. J Biol Inorg Chem **5:** 167–177

Agar JN, Krebs C, Frazzon J, Huynh BH, Dean DR, Johnson MK (2000b) IscU as a scaffold for iron-sulfur cluster biosynthesis: sequential assembly of [2Fe-2S] and [4Fe-4S] clusters in IscU. Biochemistry **39:** 7856–7862

Beinert H (2000) Iron-sulfur proteins: ancient structures, still full of surprises. J Biol Inorg Chem **5:** 2–15

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Ding H, Clark RJ (2004) Characterization of iron binding in IscA, an ancient iron-sulfur cluster assembly protein. Biochem J **379:** 433–440

Emanuelsson O, Nielsen H, Brunak S, von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol **300:** 1005–1016

Fish W (1988) Rapid colorimetric micromethod for the quantitation of complexed iron in biological samples. Methods Enzymol **158:** 357–364

Frazzon J, Fick JR, Dean DR (2002) Biosynthesis of iron-sulphur clusters is a complex and highly conserved process. Biochem Soc Trans **30:** 680–685

Gill SC, von Hippel PH (1989) Calculation of protein extinction coefficients from amino acid sequence data. Anal Biochem **182:** 319–326

Hoff KG, Silberg JJ, Vickery LE (2000) Interaction of the iron-sulfur cluster assembly protein IscU with the Hsc66/Hsc20 molecular chaperone system of *Escherichia coli*. Proc Natl Acad Sci USA **97:** 7790–7795

Jensen LT, Culotta VC (2000) Role of *Saccharomyces cerevisiae* ISA1 and ISA2 in iron homeostasis. Mol Cell Biol **20:** 3918–3927

Kato S, Mihara H, Kurihara T, Yoshimura T, Esaki N (2000) Gene cloning, purification, and characterization of two cyanobacterial NifS homologs driving iron-sulfur cluster formation. Biosci Biotechnol Biochem **64:** 2412–2419

Kaut A, Lange H, Diekert K, Kispal G, Lill R (2000) Isa1p is a component of the mitochondrial machinery for maturation of cellular iron-sulfur proteins and requires conserved cysteine residues for function. J Biol Chem **275:** 15955–15961

Keegstra K, Froehlich JE (1999) Protein import into chloroplasts. Curr Opin Plant Biol **2:** 471–476

Kessler D (2004) Slr0077 of *Synechocystis* has cysteine desulfurase as well as cystine lyase activity. Biochem Biophys Res Commun **320:** 571–577

Krebs C, Agar JN, Smith AD, Frazzon J, Dean DR, Huynh BH, Johnson MK (2001) IscA, an alternate scaffold for Fe-S cluster biosynthesis. Biochemistry **40:** 14069–14080

Lang T, Kessler D (1999) Evidence for cysteine persulfide as reaction product of L-cyst(e)ine *C-S* lyase (C-DES) from *Synechocystis*. J Biol Chem **274:** 189–195

Leon S, Touraine B, Ribot C, Briat JF, Lobreaux S (2003) Iron-sulphur cluster assembly in plants: distinct NFU proteins in mitochondria and plastids from *Arabidopsis thaliana*. Biochem J **371:** 823–830

Leon S, Touraine B, Briat JF, Lobreaux S (2002) The AtNFS2 gene from *Arabidopsis thaliana* encodes a NifS-like plastidial cysteine desulphurase. Biochem J **366:** 557–564

Lezhneva L, Amann K, Meurer J (2004) The universally conserved HCF101 protein is involved in assembly of [4Fe-4S]-cluster-containing complexes in *Arabidopsis thaliana* chloroplasts. Plant J **37:** 174–185

Lill R, Kispal G (2000) Maturation of cellular Fe-S proteins: an essential function of mitochondria. Trends Biochem Sci **25:** 352–356

Martin W, Stoebe B, Goremykin V, Hapsmann S, Hasegawa M, Kowallik KV (1998) Gene transfer to the nucleus and the evolution of chloroplasts. Nature **393:** 162–165

Morimoto K, Nishio K, Nakai M (2002) Identification of a novel prokaryotic HEAT-repeats-containing protein which interacts with a cyanobacterial IscA homolog. FEBS Lett **519:** 123–127

Morimoto K, Sato S, Tabata S, Nakai M (2003) A HEAT-repeats containing protein, IaiH, stabilizes the iron-sulfur cluster bound to the cyanobacterial IscA homologue, IscA2. J Biochem (Tokyo) **134:** 211–217

Muhlenhoff U, Gerber J, Richhardt N, Lill R (2003) Components involved in assembly and dislocation of iron-sulfur clusters on the scaffold protein Isu1p. EMBO J **22:** 4815–4825

Muhlenhoff U, Richhardt N, Ristow M, Kispal G, Lill R (2002) The yeast frataxin homolog Yfh1p plays a specific role in the maturation of cellular Fe/S proteins. Hum Mol Genet **11:** 2025–2036

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant **15:** 437–497

Nishio K, Nakai M (2000) Transfer of iron-sulfur cluster from NifU to apoferredoxin. J Biol Chem **275:** 22615–22618

Ollagnier-de-Choudens S, Mattioli T, Takahashi Y, Fontecave M (2001) Iron-sulfur cluster assembly: characterization of IscA and evidence for a specific and functional complex with ferredoxin. J Biol Chem **276:** 22604–22607

Outten FW, Djaman O, Storz G (2004) A suf operon requirement for Fe-S cluster assembly during iron starvation in *Escherichia coli*. Mol Microbiol **52:** 861–872

Outten FW, Wood MJ, Munoz FM, Storz J (2003) The SufE protein and the SufBCD complex enhance SufS cysteine desulfurase activity as part of a sulfur transfer pathway for Fe-S cluster assembly in *Escherichia coli*. J Biol Chem **278:** 45713–45719

Pilon M, Rietveld AG, Weisbeek PJ, de Kruijff B (1992) Secondary structure and folding of a functional chloroplast precursor protein. J Biol Chem **267:** 19907–19913

Pilon-Smits EAH, Garifullina GF, Abdel-Ghany S, Kato S, Mihara H, Hale KL, Burkhead JL, Esaki N, Kurihara T, Pilon M (2002)

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Characterization of a NifS-like chloroplast protein from Arabidopsis. Implications for its role in sulfur and selenium metabolism. Plant Physiol **130:** 1309–1318

Rensink WA, Pilon M, Weisbeek P (1989) Domains of transit sequence required for in vivo import in Arabidopsis chloroplast. Plant Physiol **118:** 691–699

Silberg JJ, Hoff KG, Tapley TL, Vickery LE (2001) The Fe/S assembly protein IscU behaves as a substrate for the molecular chaperone Hsc66 from *Escherichia coli*. J Biol Chem **276:** 1696–1700

Smeekens S, Baurle C, Hageman J, Keegstra K, Weisbeek P (1986) The role of the transit peptide in the routing of pre-cursors toward different chloroplast compartments. Cell **46:** 365–375

Smillie RM, Entsch B (1971) Phytoflavin. Methods Enzymol **23:** 504–514

Swofford DL (1993) PAUP: Phylogenetic Analysis Using Parsimony 3.1.1. Illinois Natural History Survey, Champaign, IL

Takahashi Y, Mitsui A, Hase T, Matsubara H (1986) Formation of the iron sulfur cluster of ferredoxin in isolated chloroplast. Proc Natl Acad Sci USA **83:** 2434–2437

Takahashi Y, Nakamura M (1999) Functional assignment of the ORF2-iscS-iscU-iscA-hscB-hscA-fdx-ORF3 gene cluster involved in the assembly of Fe-S clusters in *Escherichia coli*. J Biochem (Tokyo) **126:** 917–926

Takahashi Y, Tokumoto U (2002) A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. J Biol Chem **277:** 28380–28383

Tokumoto U, Takahashi Y (2001) Genetic analysis of the isc operon in *Escherichia coli* involved in the biogenesis of cellular iron-sulfur proteins. J Biochem (Tokyo) **130:** 63–71

Tong WH, Jameson GN, Huynh BH, Rouault TA (2003) Subcellular compartmentalization of human Nfu, an iron-sulfur cluster scaffold protein, and its ability to assemble a [4Fe-4S] cluster. Proc Natl Acad Sci USA **100:** 9762–9767

Touraine B, Boutin J, Marion-Poll A, Briat J, Peltier G, Lobreaux S (2004) Nfu2: a scaffold protein required for [4Fe-4S] and ferredoxin iron-sulfur cluster assembly in Arabidopsis chloroplasts. Plant J **40:** 101–111

Urbina HD, Silberg JJ, Hoff KG, Vickery LE (2001) Transfer of sulfur from IscS to IscU during Fe/S cluster assembly. J Biol Chem **276:** 44521–44526

Wollenberg M, Berndt C, Bill E, Schwenn JD, Seidler A (2003) A dimer of the FeS cluster biosynthesis protein IscA from cyanobacteria binds a [2Fe2S] cluster between two protomers and transfers it to [2Fe2S] and [4Fe4S] apo proteins. Eur J Biochem **270:** 1662–1671

Wu G, Mansy SS, Hemann C, Hille R, Surerus KK, Cowan JA (2002) Iron-sulfur cluster biosynthesis: characterization of *Schizosaccharomyces pombe* Isa1. J Biol Inorg Chem **7:** 526–532

Xu X, Moller S (2004) AtNAP7 is a plastidic SufC-like ATP-binding cassette/ATPase essential for Arabidopsis embryogenesis. Proc Natl Acad Sci USA **101:** 9143–9148

Yabe T, Morimoto K, Kikuchi S, Nishio K, Terashima I, Nakai M (2004) The Arabidopsis chloroplastic NifU-like protein CnfU, which can act as an iron-sulfur cluster scaffold protein, is required for biogenesis of ferredoxin and photosystem I. Plant Cell **16:** 993–1007

Ye H, Garifullina G, Abdel-Ghany S, Zhang L, Pilon-Smits EAH, Pilon M (2005) The chloroplast NifS-like protein of *Arabidopsis thaliana* is required for iron-sulfur cluster formation in ferredoxin. Planta **220:** 602–608

Yocum CF (1982) Purification of ferredoxin and plastocyanin. *In* M Edelman, RB Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier Biomedical Press, Amsterdam, pp 973–981

Yuvaniyama P, Agar JN, Cash VL, Johnson MK, Dean DR (2000) NifS-directed assembly of a transient [2Fe-2S] cluster within the NifU protein. Proc Natl Acad Sci USA **97:** 599–604

Zheng L, Cash VL, Flint DH, Dean DR (1998) Assembly of iron-sulfur clusters. Identification of an iscSUA-hscBA-fdx gene cluster from *Azotobacter vinelandii*. J Biol Chem **273:** 13264–13272

Zheng L, White RH, Cash VL, Dean DR (1994) Mechanism for the desulfurization of L-cysteine catalyzed by the nifS gene product. Biochemistry **33:** 4714–4720

Zheng L, White RH, Cash VL, Jack RF, Dean DR (1993) Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. Proc Natl Acad Sci USA **90:** 2754–2758

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Chapter 5

In this Chapter the expression of [Fe-S] biogenesis proteins was studied, and transgenic/knockout plants were constructed/screened. It was found that these biogenesis proteins are regulated by various conditions *in vivo.* All expression analysis was done by Hong Ye, as well as the construction of two plant transformation constructs (CpNifS mutant and CpSufE WT) and creation of corresponding trans-genics, and all PCR screening for knockout lines.

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Chapter 5

Investigations of the plastidic [Fe-S] biogenesis machinery: Expression analysis and preliminary studies on T-DNA insertion and over-expression lines

Hong Ye, Salah E. Abdel-Ghany, Douglas van Hoewyk, Elizabeth A.H. Pilon-Smits, and Marinus Pilon

Summary

CpNifS and CpSufE proteins are part of the plastidic [Fe-S] biogenesis machinery, probably forming a complex (see Chapter 4). This machinery supplies [Fe-S] clusters for photosynthesis. [Fe-S] clusters are also essential for nitrogen and sulfur assimilation, and may be involved in the homeostasis of iron and sulfur. To obtain more information about the role of the CpNifS-CpSufE proteins in these processes we studied expression patterns of both proteins. Preliminary data we obtained so far are shown in this chapter. It was found that both proteins are upregulated ~2-fold in the presence of excess iron and downregulated 2-fold under iron limitation, suggesting the complex is regulated by iron. The CpNifS-CpSufE expression remained the same when sulfur supply was varied up to 100-fold. However, the scaffold protein CpIscA, which is thought to function with CpNifS-CpSufE proteins, was downregulated 3-fold upon sulfur starvation. Selenium is assimilated by plants via the same pathway as sulfur, because of its similar chemical properties. The CpNifS-CpSufE proteins were upregulated 3-fold by selenate. CpNifS overexpression in plastids was found earlier to enhance selenium accumulation and tolerance in Arabidopsis. While selenium is not essential for higher plants, these results may suggest a role for the CpNifS-CpSufE

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proteins in selenium tolerance. A collection of transgenic Arabidopsis plants is currently being created, with enhanced or reduced CpNifS or CpSufE level. This collection should offer us more insight into the implications of the plastidic machinery in iron and sulfur homeostasis and selenium tolerance.

Introduction

The plastidic [Fe-S] biogenesis machinery supplies [Fe-S] clusters for a variety of proteins in chloroplasts, as schematically depicted in Figure 1 of Chapter 1. Several essential plant processes involve multiple [Fe-S] proteins, and thus are dependent on the [Fe-S] biosynthesis machinery, e.g. photosynthetic electron transport, sulfur assimilation, and nitrogen assimilation. Indeed, reduced photosynthesis caused by the impaired machinery has been shown in knockout mutants that lack the proposed [Fe-S] scaffold Nfu2 (Leon et al. 2003, Yabe et al. 2004), or the protein HCF101, which has an as yet unknown function (Lezhneva et al. 2004). Similar results were found for a CpNifS knockdown mutant (Abdel-Ghany et al. unpublished data). In this study, the focus is on expression of the [Fe-S] biosynthesis machinery regulated by iron, sulfur and selenium levels. It is feasible that this machinery plays a role in iron and sulfur homeostasis since it consumes iron and sulfur for cluster assembly, thus directly affecting iron and sulfur pools. Iron and sulfur homeostasis are important areas of study since the two elements are important for the productivity and the nutritional value of agricultural crops.

Iron is an essential micronutrient for plants. Because it is mainly present in soil as insoluble iron-oxide with low bioavailability, iron is one of the most limiting

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nutrients to plants. For a review concerning the uptake and transport of iron to leaf cells, see Curie and Briat (2003). In leaf cells, more than 90% of iron is located in chloroplasts (Terry and Abadia 1986). The chloroplast, where an [Fe-S] biogenesis machinery is present, is the major subcellular location affecting iron metabolism and homeostasis. The mechanism of iron import into chloroplasts is not clear so far, although electrochemical measurements support the hypothesis of a Fe^{2+}/H^{+} symport mechanism (Shingles et al. 2002). Within the chloroplast, ~20% of iron is associated with thylakoid membranes, and the remaining ~80% is in the stroma (Bughio et al. 1997). In thylakoid membranes, iron in the form of [Fe-S] clusters and hemes is predominantly used for electron transfer. In stroma, iron is used for assembling [Fe-S] clusters, hemes, and Fe-SOD. The excess of stromal iron is stored in ferritin proteins (Petit et al. 2001). Because iron is one of the most limiting micronutrients, plants likely employ a homeostasis mechanism to control the iron level in plastids. However, the factors/processes controlling plastidic iron homeostasis are largely unknown.

Iron transporters like IRT1 are thought to be necessary for cellular iron homeostasis of Arabidopsis (Varotto et al. 2002, Henriques et al. 2002). Also, within the chloroplast, a series of degradation and remodeling reactions of the photosynthetic apparatus is regulated in response to iron status (Moseley et al. 2002). So far it is unknown whether the [Fe-S] biogenesis machinery is regulated by iron levels in the chloroplast. The ISC-type machinery of bacteria includes IscR protein. IscR is a transcription regulator controlling the expression of the ISC machinery by sensing the cellular iron level (Schwartz et al. 2001). Conversely, some [Fe-S] biogenesis machineries can affect iron homeostasis in specific compartments. For instance, in

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mitochondria of yeast and mammalian cells, impaired [Fe-S] biogenesis results in iron overload in this compartment (for a review see Rouault and Tong 2005). The same result was observed in Arabidopsis (Kushnir et al. 2001). The mitochondrial ABC transporter Sta1 is a member of the mitochondrial [Fe-S] biogenesis machinery, mutation of which leads to accumulation of nonheme, nonprotein iron. At this point, it is unknown whether impairment of the plastidic [Fe-S] machinery affects iron homeostasis in plastids.

Sulfur is a macronutrient. Generally, it is not limiting for plants. For a recent review on sulfur metabolism in plastids see Pilon-Smits and Pilon (2005). The plastid is the predominant compartment for sulfur assimilation from sulfate to cysteine. Sulfate is activated to form adenosine-5-phosphosulfate (APS), which is subsequently reduced into sulfite and sulfide. The sulfide is finally incorporated into cysteine, which is the source of reduced sulfur in plastids. In the process of sulfate assimilation into cysteine, out of four involved enzymes, two are [Fe-S] proteins: APS reductase and sulfite reductase (SiR) (Krueger et al. 1982, Kopriva et al. 2001). Particularly SiR depends on [Fe-S] clusters. It employs a unique siroheme-[4Fe-4S] as its prosthetic group, requiring not only a direct incorporation of an [Fe-S] cluster, but also a sirohydrochlorin ferrochelatase (SirB) that is a [2Fe-2S] protein for synthesizing the siroheme (Raux-Deery et al. 2005). In addition, the SiR-catalyzed six-electron reduction requires ferredoxin, a [2Fe-2S] protein, to provide electrons. Thus, sulfur assimilation is likely highly dependent on the plastidic [Fe-S] biosynthesis machinery.

To supply reduced sulfur from cysteine, the cysteine desulfurase activity of CpNifS-CpSufE is required, which provides sulfur not only for [Fe-S] biogenesis, but

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also likely for the synthesis of various sulfur-containing biomolecules (for a review see Mihara and Esaki 2002). The machinery is essential for sulfur metabolism and possibly sulfur homeostasis in plastids, but it is an uncharted research area so far, as is the possible regulation of the machinery by sulfur levels.

Selenium is a group VIa metalloid without evident essentiality for higher plants. It is metabolized by plants via the same pathways as sulfur, because they have similar chemical properties (for a review see Terry et al. 2000). Much of selenium assimilation from selenate to seleno-cysteine takes place in chloroplasts. As described above for sulfur, selenium metabolism is likely affected by the plastidic [Fe-S] biogenesis machinery. This may affect selenium toxicity, since seleno-cysteine and seleno-methionine can be nonspecifically incorporated into proteins by replacing sulfur-containing amino acids, which results in loss of protein function (Brown and Shrift 1982). Plants thus may employ the seleno-cysteine lyase (SL) activity of NifS-like proteins to break seleno-cysteine into alanine and selenide, and avoid selenium toxicity. The plastidic [Fe-S] biosynthesis machinery, particularly CpNifS, therefore likely affects selenium accumulation and tolerance. Indeed, transgenic Arabidopsis overexpressing a mouse selenocysteine lyase (SL) displayed enhanced selenium accumulation and tolerance (Pilon et al. 2003). Arabidopsis has its own seleno-cysteine lyase activity, e.g. the CpNifS, a component of the plastidic [Fe-S] biogenesis machinery. Recently, transgenic Arabidopsis was obtained that overexpresses CpNifS in plastids. It also displayed higher selenium tolerance and accumulation (van Hoewyk et al. 2005). The transgenics showed reduced incorporation of selenium into protein, likely causing the enhanced tolerance. How the higher CpNifS level affects selenium

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uptake will require further studies. In conclusion, altering the [Fe-S] biosynthesis machinery can affect selenium metabolism. If and how the plastidic selenium level affects the [Fe-S] biosynthesis machinery is unknown.

To study whether the [Fe-S] biogenesis machinery is regulated by Fe and S nutrient status, we examined expression patterns of machinery proteins in response to varied levels of iron, sulfur and selenium. Also, to investigate the *in vivo* role of the [Fe-S] biogenesis machinery, we started the construction of a collection of transgenic plants with altered levels of these components. Together these studies should offer us more insight into the implications of the [Fe-S] biosynthesis machinery in plastid iron, sulfur and selenium metabolism.

Materials and Methods

Plant growth and treatment for expression analysis - Seeds of wild-type (WT) *Arabidopsis thaliana* (Columbia) were surface-sterilized, cold-treated at 4°C for two days, and sown on ½ strength Murashige and Skoog (MS) media (M5519, Sigma, St. Louis, MO) supplemented with 1% of sucrose (Fisher, Fair Lawn, New Jersey). Fifty seeds were sown per plate (Petri Dish, 150 x 15 mm). Plants were grown for 2 weeks at 24°C, in a 12h photoperiod at a light intensity of 40 μmol $m^{-2}s^{-1}$.

For excess iron, ferrous ammonium sulfate (Sigma, St. Louis, MO) was added to a final concentration of 0.25 mM (5-fold excess), under which seedlings showed slightly shorter shoots and less biomass. A 50- and 500-fold excess of iron proved to be lethal: the seeds did not germinate but turned black. For iron starvation, the iron chelator ferrozine (Sigma, St. Louis, MO) was added to 0.1 mM (stock

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solution is 1 M), under which seedlings grew as well as the WT, or 1 mM under which seedlings showed severely smaller shoots and shorter roots. 10 mM ferrozine proved to be lethal.

For excess sulfur, sodium sulfate (Mallinckrodt, Paris, Kentucky) was added to 3.7 mM (10-fold excess), under which seedlings grew like WT, or 37 mM (100-fold excess) under which seedlings showed slightly less shoots/less biomass. For sulfur starvation treatment, the ½ Hoagland's medium in agar was used, in which the regular sulfur concentration is 960 μM. The magnesium sulfate (Fisher, Fair Lawn, New Jersey) concentration was modulated to 50 μM (20-fold less), 25 μM (40-fold less) and 10 μM (100-fold less) under which seedlings all showed slightly smaller shoots. The magnesium concentration was maintained at a constant level by supplementation with magnesium chloride (Fisher, Fair Lawn, New Jersey).

For selenium stress, sodium selenate (Sigma, St. Louis, MO) was added to 30 μM, under which seedlings showed slightly smaller shoots and less biomass. The 300 μM selenate treatment proved to be lethal. For oxidative stress, water-dissolved paraquat (methyl viologen) (Sigma, St. Louis, MO) was added to 0.02 μM under which seedlings grew like WT, or to 0.2 μM under which seedlings showed severely smaller shoots and no roots. The 2 μM paraquat level proved to be lethal. For dark treatment, seedlings were grown from seed in the dark, under which seedlings showed severely thin yellowish stems and no leaves (etiolation).

Protein sampling and immunoblot analysis – Protein samples from total leaf homogenate were prepared and used for immunoblot analysis as described before

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(Pilon-Smits et al. 2002). All samples were taken between 1-4 pm. The software ImageJ (NIH, Bethesda, MD) was used to quantify bands.

Plant growth for transgenic lines and knockout lines – Wild-type *Arabidopsis thaliana* (Columbia) seeds were cold-treated at 4°C for two days and sown on soil (200 seeds per pot). Plants were grown at room temperature and a 10h photoperiod for 8 weeks. Then they were moved to grow in a photoperiod of 16h for 1 week until abundant flowers were available, and a floral dip was done to create transgenic plants, as described by Pilon et al. (2003) and below. After the floral dip, the plants were grown at a 16h photoperiod until they were harvested for seeds.

All knockout seeds for both CpNifS and CpSufE were originally sown on soil, but we could not find any homozygous lines. Therefore we switched to sucrose agar medium. To screen for homozygous CpSufE knockout lines, Arabidopsis seeds of Salk_011580 line (ABRC, Columbus, OH) were surfaced-sterilized and cold-treated at 4°C for four days, followed by being sown on full-strength Murashige and Skoog (MS) media (Sigma, St. Louis, MO) supplemented with 3% of sucrose (Fisher, Fair Lawn, New Jersey). Four seeds were sown per Magenta box. Plants were grown at 24 °C and a 12h photoperiod for 4 weeks. DNA samples were extracted by shorty DNA isolation and checked by PCR. The primers used were LBb1 (5'-GCGTGGACCG CTTG CTGCAA CT-3') and SufE-RP (5'-GCTCCTTCTCCAATTTCTCTCT-3') for the T-DNA insert, and SufE-mature-Nco (5'-CATGCCATGGCGGCTT CATCAT CTCCG TCG-3') and SufE-RP for the wildtype gene. A PCR program with a 55°C annealing temperature (45 seconds) and a 72°C extension temperature (1 min) for 30 rounds was used. The WT PCR product was 680 bp, while the T-DNA product was

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450 bp. For the CpNifS knockout lines, Arabidopsis seeds of Salk_023084 and Salk_021630 lines (ABRC, Columbus, OH) were used. The plants were grown as described above and screened for homozygous lines using PCR. Primers used for Salk_021630 are NifS30-RP (5'-TGTCTTCCCTGCCTGATGTGC-3') and NifS30-LP (5'-AAACCAGCCGAGAGAT CCAGC-3') for the wildtype gene, LBb1 and NifS30-RP or LP for the T-DNA insert. Primers used for Salk_023084 are NifS84-LP (5'-TCTGCAAAGCGTCCAATACAGC-3') and NifS84-RP (5'-GACACAAGATGAA CAAGAA GGCGA-3') for the wildtype gene, and LBb1 and NifS84-LP or RP for the T-DNA insert. The PCR program used had 30 rounds of 55°C annealing temperature 45 seconds and 72°C extension temperatrure 1 min.

Plasmid construction for overexpressing full-length CpNifS and CpSufE of both WT and point mutants – The plasmid construction for overexpressing CpNifS was described by Van Hoewyk et al. (2005). The vector pFGC5941 (Chromdb database) was used for overexpression in this study. It contains the omega leader of the TMV between the promoter sequence and the gene of interest. This sequence is thought to increase translation efficiency. This vector also contains a poly-adenylation signal sequence at the end of the insert.

For overexpression of the full-length $CpNifS_{C388S}$ mutant, a site-directed mutagenesis with two rounds of PCR was performed. In the first round of PCR, two fragments were amplified with primers T7 (5'-CGAAATTAATACG ACTCACTA TAG-3') and $NifS_{C388S}$-R (5'-TGGAGTGGCTGTGCGGAGTG GTGTCC TGAC CTT-3'), and primers T7 terminator (5'-TGCTAGTTATTGCTCAGCGGT-3') and $NifS_{C388S}$-F (5'-AAGGTCAGGACACCACTCCGCA CAGCCACTCCA-3'), using

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the plasmid pPNFS-8 containing the full-length CpNifS sequence (Pilon-Smits et al. 2002) as a template. In the second round of PCR, the two fragments were used together as template and amplified with primers NifS-precursor (5'-CATGCCATGG AAGGTGT GGCTAT GAAACTC-3' with a NcoI site underlined) and NFS-B2 (5'-TCGCCGGATCCACTT ATTTGAAAGAGTTGAA -3' with a BamHI site underlined). The final PCR product (1.6 kb) was digested with *Nco*I and *BamH*I, and ligated into vector pFGC5941 (Chromdb Database), leading to pFGC-$PNFS_{C388S}$.

For overexpression of the full-length CpSufE, the precursor sequence was amplified with primers SufE-precursor (5'-CATGCCATGGCAGCAGCGATGT CTTCTTC-3' with a NcoI site underlined) and SufE-Bam (5'-CGGGATCCTCAA ACCTCAGCAGGAGTCT-3' with a BamHI site underlined), using the plasmid pPrSufE (see Chapter 4) as a template. The amplified fragment (1.1 kb) was digested with NcoI and BamHI, and ligated into vector pFGC5941, leading to pFGC-PSUFE.

For overexpression of the full-length $CpSufE_{C65S}$ mutant, a site-directed mutagenesis with two rounds of PCR was performed. In the first round of PCR, two fragments were amplified with primers T7 and $SufE_{C65S}$-R (5'-CCAAACCTGAGAA ACAGATCCTTCTACT TTATT-3'), and primers T7 terminator and $SufE_{C65S}$-F (5'-AATAAAGTAGAAGGATCTGTTTCTCAGGTTTG G-3'), using the plasmid pPrSufE as a template. In the second round of PCR, the two fragments were used together as template and amplified with primers SufE-precursor and SufE-Bam. The final PCR product (1.1 kb) was digested with *Nco*I and *BamH*I, and ligated into vector pFGC5941, leading to pFGC-$PSUE_{C65S}$. Constructs were transformed into *E. coli* strain DH5α. All constructs were verified by DNA sequencing. Constructs were

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finally transformed into Agrobacterium strain C58C1 (selection for kanamycin resistance on LB medium). The plasmids used for transformation were prepared using the Plasmid Mini Kit (Qiagen, Valencia, CA).

Transformation of Arabidopsis thaliana – The wild-type *Arabidopsis thaliana* (Columbia) was transformed with *Agrobacterium tumefaciens* strain C58C1 carrying pFGC5941 constructs, by the floral dip method (Clough and Bent 1998). Transgenic lines can be selected for Basta (glufosinate) resistance, followed by examination by PCR and immunoblot.

Results

CpSufE is upregulated in the CpNifS overexpressor – The transgenic Arabidopsis plants that overexpress wild-type CpNifS in their chloroplasts, named CpNifS P-lines, were obtained as described by Van Hoewyk et al. (2005). As described in Chapter 4, CpSufE was found to form a complex with CpNifS and activate its cysteine desulfurase (cysD) activity, likely constituting a novel two-component cysD. In order to check if the CpSufE protein expression level is upregulated in the CpNifS P-lines, homogenate protein sample from P-line #55 was applied in an immunoblot (Figure 1). A homogenate protein sample from wild-type (WT) plants was used as a control. Actin, a highly conserved and constitively expressed cytosolic protein, was used as loading control. The similar stain of actin bands indeed indicated equal loading of all samples. CpNifS was estimated to be upregulated 7-fold in the CpNifS#55 plants compared to WT, as quantified with the ImageJ software. Intriguingly, CpSufE was upregulated 2.5-fold in line CpNifS#55

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compared to WT. These results suggest that CpSufE is also upregulated in the CpNifS overexpressor. Note: This experiment was performed once. More repeats are required using multiple CpNifS overexpressor lines.

The chloroplastic [Fe-S] biogenesis machinery is upregulated by oxidative stress and light – The SUF-type [Fe-S] biogenesis system in bacteria is responsible for [Fe-S] cluster biosynthesis under oxidative stress and iron limitation (Nachin et al. 2001, Outten et al. 2004). Its expression can be induced by oxidative stress. The chloroplastic [Fe-S] biogenesis machinery is similar to the bacterial SUF system (see Chapter 1), but an oxidative stress-induced expression has never been shown for the chloroplastic SUF-like machinery. In addition, the machinery supplies clusters for the photosynthetic electron transport chain. A light-regulated expression of the machinery is likely, but remains to be investigated. To obtain this information about regulation of expression, the chloroplastic [Fe-S] machinery components were subjected to expression analysis in response to oxidative stress and light induction. Preliminary results are shown in Figure 2. Paraquat was used to introduce oxidative stress within plant cells. The similar stain of actin bands indicated equal loading. Both CpNifS and CpSufE were upregulated ~ 2-fold by oxidative stress at the protein level. In the light-dark experiment, the CpNifS level in dark-grown plants was 3-fold less than that in the control grown under the regular photoperiod, and the CpSufE level was 4-fold lower in dark-grown plants. This indicates the CpNifS-CpSufE complex is induced by light. This latter result was confirmed by at least two other trials. Note: The paraquat experiment was performed once, so more repeats are required.

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CpNifS and CpSufE are downregulated under iron limitation and upregulated in the presence of excess iron – To test the influence of iron supply on CpNifS/CpSufE expression, plants were grown at different iron concentrations. The iron chelator ferrozine was added to the media to induce iron starvation, and excess Fe was provided in the form of ferrous ammonium sulfate. The similar intensity of the actin bands indicated equal loading (Figure 3). Under iron limitation, expression of CpNifS and CpSufE was reduced 2-fold. The addition of more ferrozine, reducing bioavailable iron, resulted in less expression. Similarly, lower CpNifS expression was found in seedlings grown on Hoagland's agar media under iron limitation (Appendix 1), which is 2.4 μM ferrous salt (10-fold less than the regular concentration). In the presence of excess iron, which is 0.25 mM ferrous salt in MS media (5-fold more than the regular concentration), the expression of CpNifS and CpSufE appeared to be upregulated ~ 2-fold (data not shown, the blot was not clean). Taken together, these preliminary results suggest that the CpNifS-CpSufE proteins are regulated by iron level. The CpIscA immunoblot did not work for unknown reason.

CpIscA is downregulated under sulfur starvation – To test the effect of sulfur, the [Fe-S] machinery components CpNifS, CpSufE and CpIscA were subjected to an expression analysis in response to sulfur supply. An excess of sulfur in the form of sulfate did not alter the expression of CpNifS, CpSufE and CpIscA (data not shown). Under sulfur starvation, expression of CpNifS and CpSufE also did not change. However, when the sulfate concentration was lowered to 25 μM (40-fold less than the regular concentration) or even 10 μM (100-fold less), CpIscA expression was downregulated 3-fold (Figure 4). These results suggest that this molecular scaffold of

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the [Fe-S] biogenesis machinery is regulated by sulfur supply, while the cysteine desulfurase complex is not evidently affected within this range of sulfur supply. Note: More repeats are required to verify this finding, as these experiments were performed once.

CpNifS and CpSufE are upregulated in response to selenate – To test the effect of selenium, we examined expression of CpNifS, CpSufE and CpIscA in response to selenium feeding. The expression of CpIscA did not show a clear difference with or without selenate (Figure 5). However, the expression of CpNifS and CpSufE was upregulated ~ 3-fold in response to selenate, indicating the machinery is induced by selenium. The similar stain of actin bands verified equal loading. An upregulation of CpNifS in the presence of 30μM selenate was also observed in seedlings grown on Hoagland's media (Appendix 1). Once again, more repeats are required.

Transgenic Arabidopsis overexpressing CpNifS or CpSufE in chloroplasts – In a different approach to investigate whether an altered chloroplastic [Fe-S] machinery affects selenium metabolism, the above-mentioned CpNifS overexpressing P-lines were tested for selenium accumulation and tolerance. It was found that CpNifS overexpression resulted in selenium accumulation and tolerance in plants (Van Hoewyk et al. 2005).

In order to investigate whether iron/sulfur/selenate metabolism is affected in other transgenic plants, WT or mutant inactive CpNifS or CpSufE proteins were overexpressed in Arabidopsis chloroplasts. A binary vector pFGC5941 (Chromdb Database) was used to subclone these genes (Figure 6). CpNifS protein has five

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cysteine residues, each of which has been mutated to a serine and expressed in *E.coli.* (data not shown). The fifth cysteine (cys388 in the mature sequence) is essential for its cysteine desulfurase acitivity but not for selenocysteine lyase activity or the ability of binding CpSufE (Chapter 3). The full-length CpNifS sequence with a mutation at the cys388 was overexpressed in Arabidopsis chloroplasts, resulting in putative CpNifS dominant negative lines as described in the Materials and Methods. The full-length CpSufE sequence was overexpressed in Arabidopsis chloroplasts, resulting in the CpSufE P-lines. CpSufE has only one cysteine residue (cys65 in the mature sequence), which is essential for its activity to stimulate the cysteine desulfurase of CpNifS but not for the ability to bind CpNifS (Chapter 3). The full-length CpSufE sequence with a mutation at the cys65 was also overexpressed in Arabidopsis chloroplasts, resulting in CpSufE dominant-negative lines. All constructs were sequenced and transformed into plants. The resulting seeds will be selected for Basta resistance.

Knockout mutants of CpNifS and CpSufE – In other studies, impaired mitochondrial [Fe-S] biogenesis resulted in iron overload in mitochondria (Kushnir et al. 2001). Knockout mutants may also be a useful tool to investigate implications of the plastidic [Fe-S] machinery in the homeostasis of iron and sulfur. Arabidopsis lines of available CpNifS and CpSufE knockouts (T-DNA insertion sites are illustrated in Figure 7A) were grown on soil and screened for any viable homozygous knockout line. However, when PCR was used to identify the T-DNA insertion within the CpNifS genomic sequence, no plants carrying the T-DNA fragment (either line) could be identified. All samples only showed the presence of the WT CpNifS fragment, indicating that they were all homozygous WT Arabidopsis lines. The failure to

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identify a homozygous CpNifS knockout line or even a heterozygous line is likely because the homozygous knockout is lethal and the heterozygous plants got lost during the repeated selfing of the knockout lines, which is known to lead to homozygosity at >95% of all loci after the 6th generation. Therefore, in an alternative approach to reduce CpNifS expression, CpNifS knockdown lines were obtained using constitutive or inducible RNAi constructs, which are currently being characterized (Abdel-Ghany et al. unpublished data).

In the CpSufE knockout screen, heterozygous lines were found. Seeds were collected and sown on soil for the next generation. Fifty-two seeds were sown on soil. Only twenty-eight seeds germinated and grew into mature plants, none of which displayed any phenotype. In PCR, the T-DNA was identified in 19 samples out of the 28 plants, which all also had the WT fragment, indicating they were heterozygous. The other 9 plants had only the WT fragment, and therefore were WT plants. Judged from a chi-square test, these results correspond with a 2:1 ratio of heterozygotes to wildtype. Normally a 1:2:1 segregation would be expected for the offspring from a single insert T-DNA heterozygote, but no homozygotes were observed likely because homozygosity was lethal on soil. In a different attempt to get viable homozygous CpSufE knockout plants, seeds from a heterozygous knockout plant were sown on full MS media supplemented with sucrose. A few homozygous CpSufE knockout lines, much less than the expected number, were identified using PCR. They showed a severe phenotype even in the presence of sucrose (Figure 8B, top), and died shortly after germination. Their heterozygous siblings did not show any phenotype (Figure 7B, bottom). Taken together, it is likely that the homozygous CpSufE knockout

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displays embryonic lethality on soil. As an alternative strategy to study the phenotype of plants with reduced CpSufE levels, a knockdown RNAi strategy may be used, which proved to be successful for CpNifS (Abdel-Ghany et al. unpublished data).

Discussion

Since these expression analysis data are preliminary (most experiments were performed once), only tentative conclusions can be drawn and discussed in this part. CpNifS and CpSufE appeared to be upregulated in the presence of excess iron, and downregulated under iron limitation. The CpIscA protein was downregulated under sulfur starvation, while CpNifS and CpSufE expression was not affected by sulfur supply. CpNifS and CpSufE were upregulated in response to selenium. These results suggest that components of the [Fe-S] cluster biogenesis machinery are regulated in response to Fe, S, and the related element Se. Conversely, they may affect homeostasis of these elements. More information on the effects of altering levels of these [Fe-S] biosynthesis proteins on iron, sulfur and selenium metabolism can be expected with the transgenic lines and knockout mutants that are currently being investigated.

CpSufE was upregulated in the transgenic plants overexpressing CpNifS. Perhaps CpSufE is stabilized by binding to CpNifS. This would be in agreement with the finding that the two proteins form a cysteine desulfurase complex in a 1:1 ratio *in vitro* (see Chapter 3). A NifS-like cysteine desulfurase is conserved in all [Fe-S] cluster biogenesis systems identified so far. The CpNifS in the chloroplast stroma is required for the [Fe-S] formation in this compartment (Ye et al. 2005), highlighting the importance of this protein. CpSufE was found to 20-fold stimulate CpNifS-

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mediated [Fe-S] formation. The CpNifS-CpSufE complex is therefore a key component of the plastidic [Fe-S] biogenesis machinery. The complex was found to be oxidative stress-induced, suggesting a role of the plastidic [Fe-S] biogenesis machinery in repair of [Fe-S] clusters impaired by oxidative stress. It was also light-induced, consistent with a role in photosynthesis.

Iron is a limiting micronutrient for plants, requiring a controlled homeostasis *in vivo*. The concentration range allowing normal plant growth is relatively narrow. Indeed, in this study the treatment of 5-fold excessive iron was harmful but allowed plants to grow normally, displaying a mild inhibition (see Materials and Methods). The treatment with 1 mM ferrozine (or 10-fold less iron) inhibited plant growth more severely. Interestingly, a more excessive or more limited iron supply resulted in a similar response, namely no seed germination and black coloration of the seeds. In the examination of the [Fe-S] machinery in response to varied iron levels, the CpNifS-CpSufE complex was observed to be upregulated by excess iron and downregulated by iron limitation, while CpIscA was not affected. These results may be indicative of an involvement of the CpNifS-CpSufE complex in plastidic iron homeostasis. However, the nature of this role remains to be characterized. The various CpNifS/CpSufE transgenic plants should be useful for a further iron homeostasis study.

Sulfur is a macronutrient that is rarely limiting. Indeed, in this study a 100-fold excess or 100-fold limitation of sulfur supply only resulted in a slight phenotype (see Materials and Methods). The [Fe-S] machinery not only directly utilizes sulfur for [Fe-S] cluster assembly, but also supplies [Fe-S] clusters for enzymes in sulfur

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assimilation. A relationship between the machinery and sulfur homeostasis therefore may be expected. However, the CpNifS-CpSufE complex was found not to change over a wide range of sulfur concentrations. Perhaps these sulfur treatments were not stressful enough, as judged from the slight phenotype, to force plants to change the activity of CpNifS-CpSufE, which is probably constitutive under normal physiological conditions. Interestingly, the scaffold molecule CpIscA was found to be reduced 3-fold under sulfur starvation, suggesting a more tight connection between this scaffold and sulfur homeostasis. However, more experiments are needed to better understand the regulatory mechanisms involved, and their functions.

Selenium is a non-essential but highly toxic element. Plants take up selenium inadvertently because of its similarity to sulfur. Because of its toxicity, a tightly controlled selenium detoxification mechanism is expected to be present in plants. Indeed in this study, only 30 μM selenate treatment led to a similar level of phenotype caused by ~ 300 μM iron. Selenium at 300 μM was already lethal. Selenium is though to be assimilated by the same pathway as sulfur, which heavily depends on [Fe-S] cluster supply for activities. Presumably, an enhanced [Fe-S] biosynthesis machinery can increase in plants the ability to reduce selenate, which may play a role in its detoxification. Indeed, the transgenic Arabidopsis overexpressing CpNifS in plastids displayed higher selenium accumulation and tolerance (Van Hoewyk et al. 2005). This likely was mainly due to the direct activity of the CpNifS selenocysteine lyase activity, which breaks down selenocysteine into elemental selenium and alanine, thus preventing incorporation into proteins. Indeed, the CpNifS-CpSufE complex was found to be upregulated ~3-fold by selenium, suggesting the selenocysteine lyase

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activity of the complex contributes to plastidic selenium detoxification. To date, this is the only direct evidence to support a role for (part of) the [Fe-S] biogenesis machinery in plastidic selenium metabolism.

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References

Brown, T.A., and Shrift, A. (1982) Selenium: toxicity and tolerance in higher plants. *Biol. Rev.* 57, 59-84

Bughio, N., Takahashi, M., Yoshimura, E., Nishizawa, N.K., and Mori, S. (1997) Light-dependant iron transport into isolated barley chloroplasts. *Plant Cell Physiol.* 38, 101-105

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformations of Arabidopsis thaliana. *Plant J.* 16, 735-743

Curie, C., and Briat, J.F. (2003) iron transport and signaling in plants. *Annu. Rev. Plant Biol.* 54, 183-206

Henriques R, Jasik J, Klein M, Martinoia E, Feller U, Schell J, Pais MS, Koncz C. Knock-out of Arabidopsis metal transporter gene IRT1 results in iron deficiency accompanied by cell differentiation defects. Plant Mol Biol. 2002 Nov;50(4-5):587-97.

Kopriva, S., Buchert, T., Fritz, G., Suter, M., Weber, M., Benda, R., Schaller, J., and Feller, U., Schurmann, P., Schunemann, V., Trautwein, A.X., Kroneck, P.M., and Brunold, C. (2001) Plant adenosine 5'-phosphosulfate reductase is a novel iron-sulfur protein. *J. Biol. Chem.* 276, 42881-42886

Krueger, R.J., and Siegel, L.M. (1982) Spinach siroheme enzymes: Isolation and characterization of ferredoxin-sulfite reductase and comparison of properties with ferredoxin-nitrite reductase. *Biochemistry* 21, 2892-2904

Kushnir, S., Babiychuk, E., Storozhenko, S., Davey, M.W., Papenbrock, J., De Rycke, R., Engler, G., Stephan, U.W., Lange, H., Kispal, G., Lill, R., and Van Montagu, M. (2001) A Mutation of the Mitochondrial ABC Transporter Sta1 Leads to Dwarfism and Chlorosis in the Arabidopsis Mutant *starik. Plant cell* 13, 89-100

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Leon, S., Touraine, B., Ribot, C., Briat, J.F., and Lobreaux, S. (2003) Iron-sulphur cluster assembly in plants: distinct NFU proteins in mitochondria and plastids from Arabidopsis thaliana. *Biochem. J.* 371, 823-830

Lezhneva, L., Amann, K., and Meurer, J. (2004) The universally conserved HCF101 protein is involved in assembly of [4Fe-4S]-cluster-containing complexes in Arabidopsis thaliana chloroplasts. *Plant J.* 37, 174-185

Mihara, H., and Esaki, N. (2002) Bacterial cysteine desulfurases: their function and mechanisms. *Appl. Microbiol. Biotechnol.* 60, 12-23

Moseley, J.L., Allinger, T., Herzog, S., Hoerth, P., Wehinger, E., Merchant, S. and Hippler, M. (2002) Adaptation to Fe-deficiency requires remodeling of the photosynthetic apparatus. *EMBO J,* 21, 6709-6720.

Nachin, L., El Hassouni, M., Loiseau, L., Expert, D., and Barras, F. (2001) SoxR-dependant response to oxidative stress and virulence of Erwinia chrysanthemi: the key role of SufC, an orphan ABC ATPase. *Mol. Microbiol.* 39, 960-972

Outten, F.W., Djaman, O., and Storz, G. (2004) A suf operon requirement for [Fe-S] cluster assembly during iron starvation in Escherichia coli. *Mol. Microbiol.* 52, 861-872

Petit, J.M., Briat, J.F., and Lobreaux, S. (2001) Structure and differential expression of the four members of the Arabidopsis thaliana ferritin gene family. *Biochem. J.* 359, 575-582

Pilon, M., Owen, J.D., Garifullina, G.F., Kurihara, T., Mihara, H., Esaki, N., and Pilon-Smits, E.A. (2003) Enhanced selenium tolerance and accumulation in transgenic Arabidopsis expressing a mouse selenocysteine lyase. *Plant Physiol.* 131, 1250-1257

Pilon-Smits, E.A.H., Garifullina, G.F., Abdel-Ghany, S., Kato, S., Mihara, H., Hale, K.L., Burkhead, J.L., Esaki, N., Kurihara, T., and Pilon, M. (2002) Characterization of a NifS-like chloroplast protein from Arabidopsis. Implications for its role in sulfur and selenium metabolism. *Plant Physiol.* 130, 1309-1318

Pilon-Smits, E.A.H., and Pilon, M. (2005) Sulfur metabolism in plastids. In: Wise, R.R., Hoober, J.K. (Eds) "*Advances in Photosynthesis and Respiration - The Structure andFunction of Plastids*". Kluwer Academic Publishers, Dordrecht, The Netherlands. In press

Raux-Deery, E., Leech, H.K., Nakrieko, K.A., McLean, K.J., Munro, A.W., Heathcote, P., Rigby, S.E., Smith, A.G., and Warren, M.J. (2005) Identification and characterization of the terminal enzyme of siroheme biosynthesis from Arabidopsis thaliana: a plastid-located sirohydrochlorin ferrochelatase containing a 2FE-2S center. *J. Biol. Chem.* 280, 4713-4721

Rouault, T.A. and Tong, W.H. (2005) Iron-sulphur cluster biogenesis and mitochondrial iron homeostasis. *Nat. Rev. Mol. Cell. Biol.* 6, 345-351

Schwartz, C.J., Giel, J.L., Patschkowski, T., Luther, C., Ruzicka, F.J., Beinert, H., and Kiley, P.J. (2001) IscR, an [Fe-S] cluster-containing transcription factor, represses expression of *Escherichia coli* genes encoding [Fe-S] cluster assembly proteins. *Proc. Natl. Acad. Sci. USA.* 98, 14895-14900

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Shingles, R., North, M., and McCarty, R.E. (2002) ferrous iron transport across chloroplast inner envelope membranes. *Plant Physiol.* 128, 1022-1030

Terry, N., and Abadia, J. (1986) Function of iron in chloroplasts. *J. Plant Nutr.* 9, 609-646

Terry, N., Zayed, A.M., de Souza, M.P., and Tarun, A.S. (2000) Selenium in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 51, 401-432

Van Hoewyk, D., Garifullina, G.F., Ackley, A.R., Abdel-Ghany, S.E., Marcus, M.A., Fakra, S., Ishiyama, K., Ioue, E., Pilon, M., Takahashi, H., and Pilon-Smits, E.A.H. (2005) Overexpression of AtCpNifS enhances selenium tolerance and accumulation in Arabidopsis. *Plant Physiol.* In press

Varotto C, Maiwald D, Pesaresi P, Jahns P, Salamini F, Leister D. (2002) The metal ion transporter IRT1 is necessary for iron homeostasis and efficient photosynthesis in Arabidopsis thaliana. *Plant J.* 31, 589-599

Yabe, T., Morimoto, K., Kikuchi, S., Nishio, K., Terashima, I., and Nakai, M. (2004) The Arabidopsis chloroplastic NifU-like protein CnfU, which can act as an iron-sulfur cluster scaffold protein, is required for biogenesis of ferredoxin and photosystem I. *Plant Cell* 16, 993-1007

Ye, H., Garifullina, G.F., Abdel-Ghany, S.E., Zhang, L., Pilon-Smits, E.A., and Pilon, M. (2005) The chloroplast NifS-like protein of Arabidopsis thaliana is required for iron-sulfur cluster formation in ferredoxin. *Planta* 220, 602-608

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Figure legends

Figure 1. CpSufE is co-upregulated in the CpNifS over-expressor. Arabidopsis (Columbia) of either wild type (WT) or CpNifS over-expressor #55 (Van Hoewyk et al. 2005) was grown on soil, at 25 °C, in 8 hours light/16 hours dark, for 4 weeks. Shoot protein samples were prepared. Protein concentrations were determined using the Bradford assay. 20 μg of total protein for each sample was separated on a 12.5% SDS-PAGE gel, blotted onto a 0.2 μm nitrocellulose membrane, and stained with specific antibodies. Actin was used as a control to verify equal loading.

Figure 2. *A.* CpNifS and CpSufE are upregulated under oxidative stress. Wild type Arabidopsis (Columbia) was grown on ½ MS media, supplied with 0 μM, 0.02 μM or 0.2 μM paraquat at 24 °C, in a light period of 12 hours light/12 hours darkness for 2 weeks. Shoot protein samples were prepared. Protein concentrations were determined using the Bradford assay. Samples (20 μg) were separated on a 12.5% SDS-PAGE gel, blotted onto a 0.2 μm nitrocellulose membrane, and stained with specific antibodies. Actin was used as a control to verify equal loading. *B.* CpNifS and CpSufE are expressed at a lower level in the darkness. Wild type Arabidopsis (Columbia) was grown on ½ MS media at 24 °C, for 2 weeks. Dark: continuous darkness. Light: 12 hour photoperiod. Shoot protein samples (20 μg) were prepared, separated and analyzed by Western blotting as described for Figure 2 and in Materials and Methods.

Figure 3. CpNifS and CpSufE are downregulated under iron limitation. Wild type Arabidopsis (Columbia) was grown on ½ MS media, to which 0 mM, 0.1 mM or 1

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mM ferrozine was added, at 24 °C, in a light period of 12 hours light/12 hours darkness, for 2 weeks. Shoot proteins were prepared and analyzed by Western blotting as described for Figure 2 and in Materials and Methods.

Figure 4. CpIscA is downregulated under sulfur starvation. Wild type Arabidopsis (Columbia) was grown on Hoagland's media (in agar) in which the sulfate concentration was modulated, at 24 °C, in a light period of 12 hours light/12 hours darkness, for 2 weeks. Control corresponds with a sulfate concentration of 960 μM. Shoot proteins were prepared and analyzed by Western blotting as described for Figure 2 and in Materials and Methods.

Figure 5. CpNifS and CpSufE are upregulated in response to selenate treatment. Wild type Arabidopsis (Columbia) was grown on ½ MS media in which the selenate concentration was either 0 or 30 μM, at 24 °C, in a light period of 12 hours light/12 hours darkness, for 2 weeks. Shoot proteins were prepared and analyzed by Western blotting as described for Figure 2 and in Materials and Methods.

Figure 6. Vector map of pFGC5941 (Chromdb Database). Km: kanamycin resistance. LB and RB: Left Border and Right Border. The LB and RB-flanking fragment mediates insertion into the plant genome. BAR: Basta Resistance. CaMV 35S promoter: Cauliflower mosaic virus 35S promoter. The digestion sites of *Nco*I and *BamH*I were used to subclone genes (all are full-length precursors) in this study.

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Figure 7. *A*. Maps of genomic sequences of CpSufE (top) and CpNifS (bottom) showing the predicted T-DNA insertions. In the CpSufE genomic sequence, the box labeled with Salk-011580 indicates the T-DNA insert. The box labeled with number 1 is the exon, which is 1100 bp long. Lines labeled with letters are non-transcribed regions. a: 70 bp, b: 250 bp. In the CpNifS genomic sequence, boxes labeled with Salk-023084 and Salk-021630 represent two different T-DNA inserts, respectively. Boxes in numbers are exons. Length for each from 1 to 9 is: 200, 300, 180, 140, 70, 60, 100, 160, and 170 bp. Lines in letters are introns. Length for each from a to j is: 40, 100, 80, 110, 100, 120, 80, 100, 520, and 320 bp. *B*. Photo of CpSufE knockout plants. Plants were grown on full MS media supplemented with sucrose. A homozygous CpSufE knockout plant (top) and a heterozygous CpSufE knockout plant (bottom) are shown.

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Figure 1

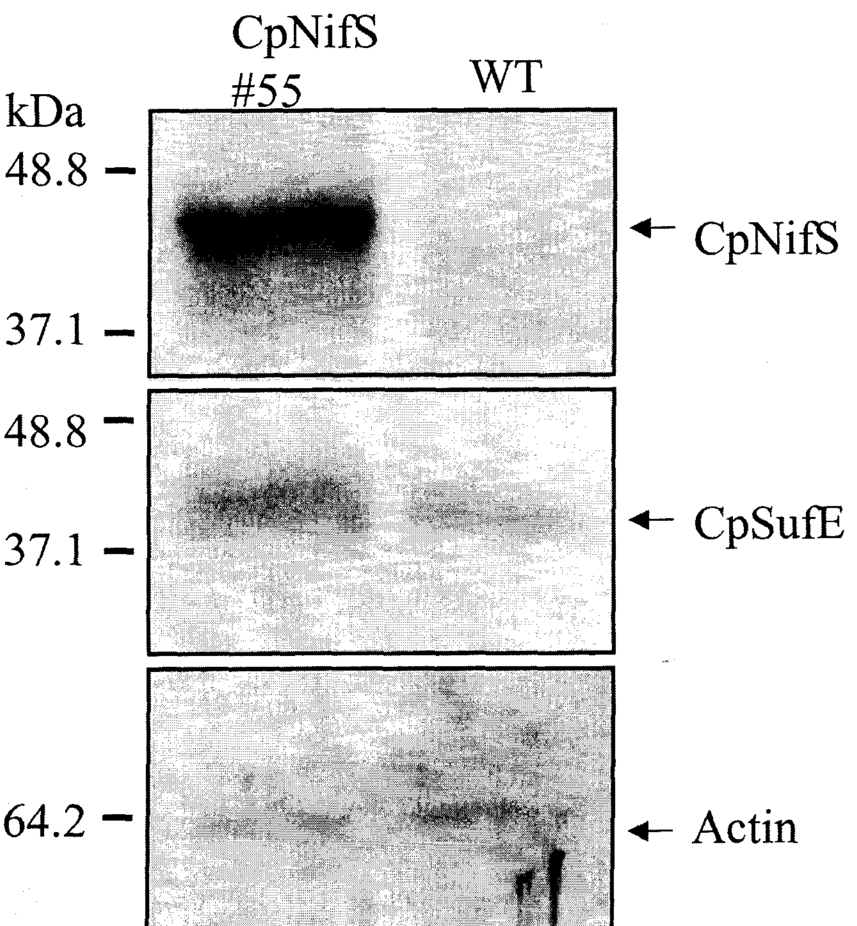

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Figure 2

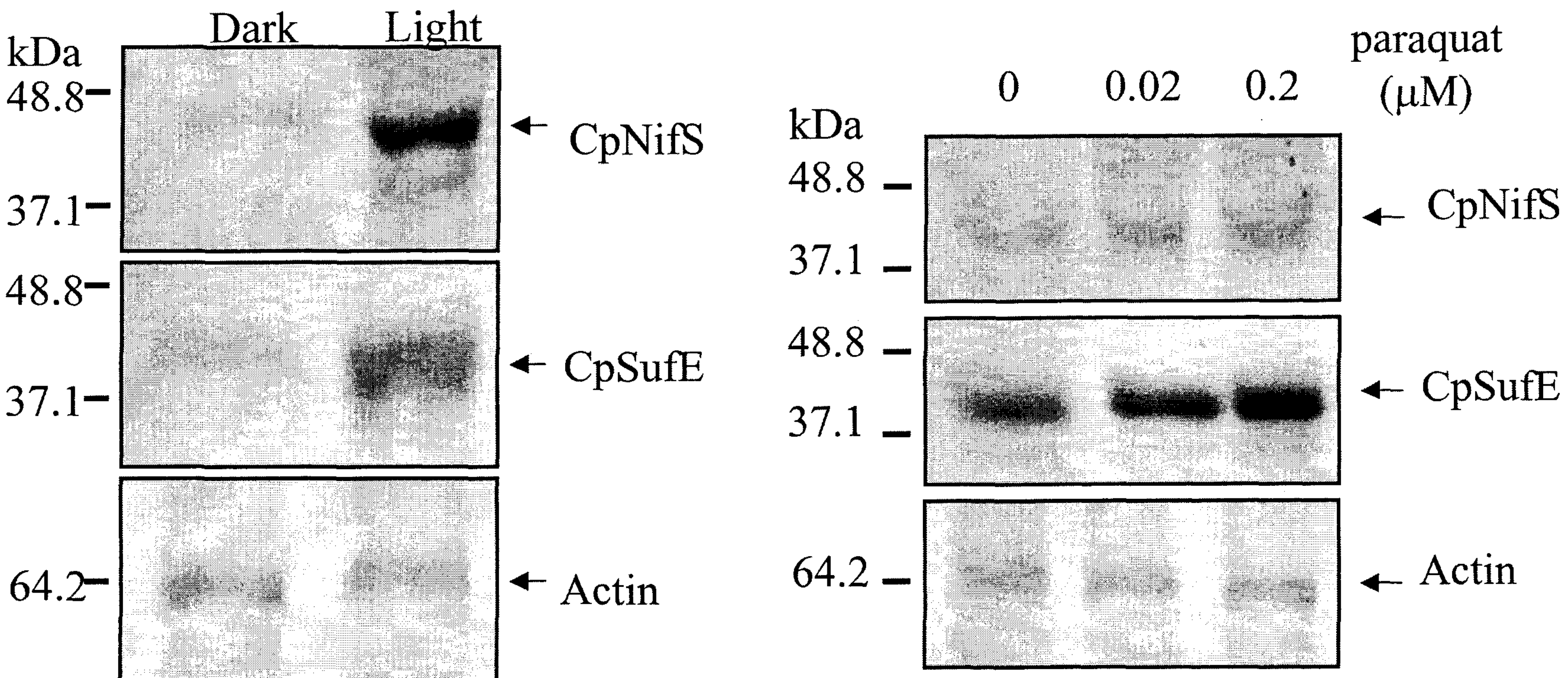

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Figure 3

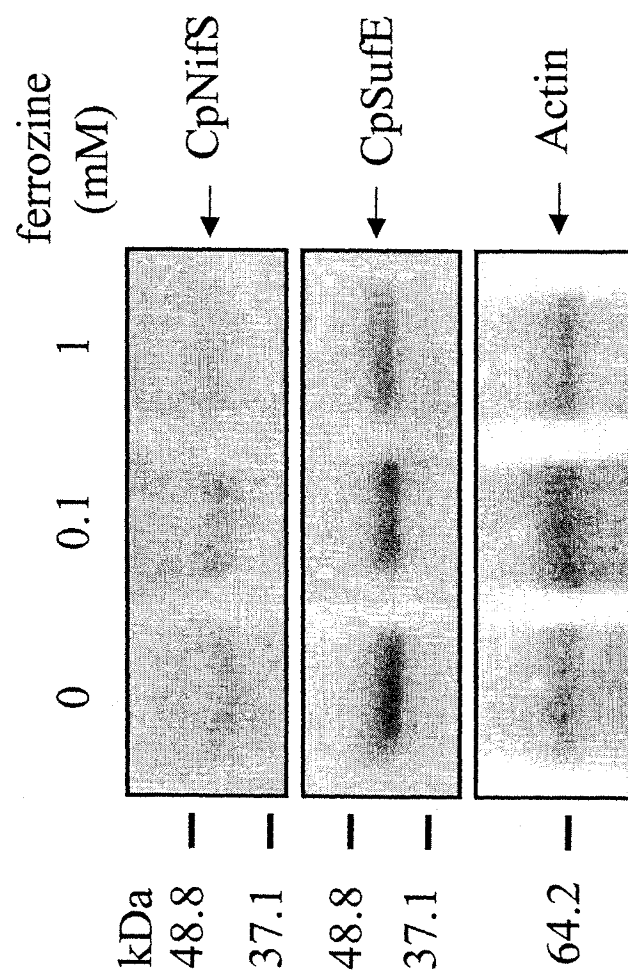

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Figure 4

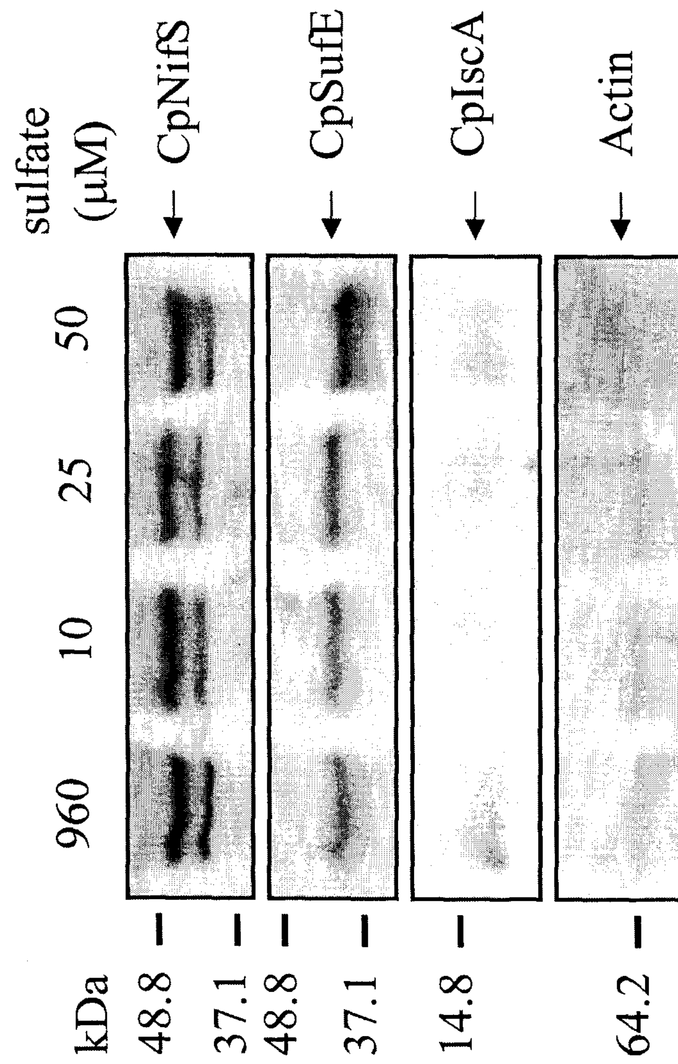

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Figure 5

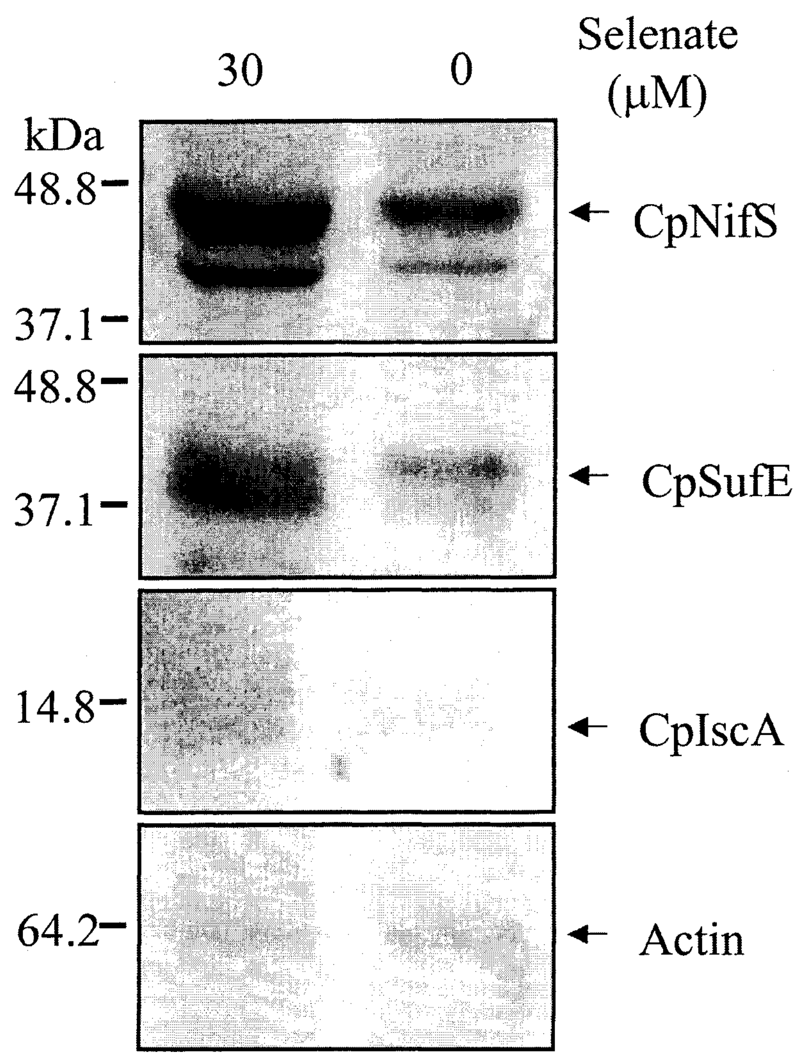

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Figure 6

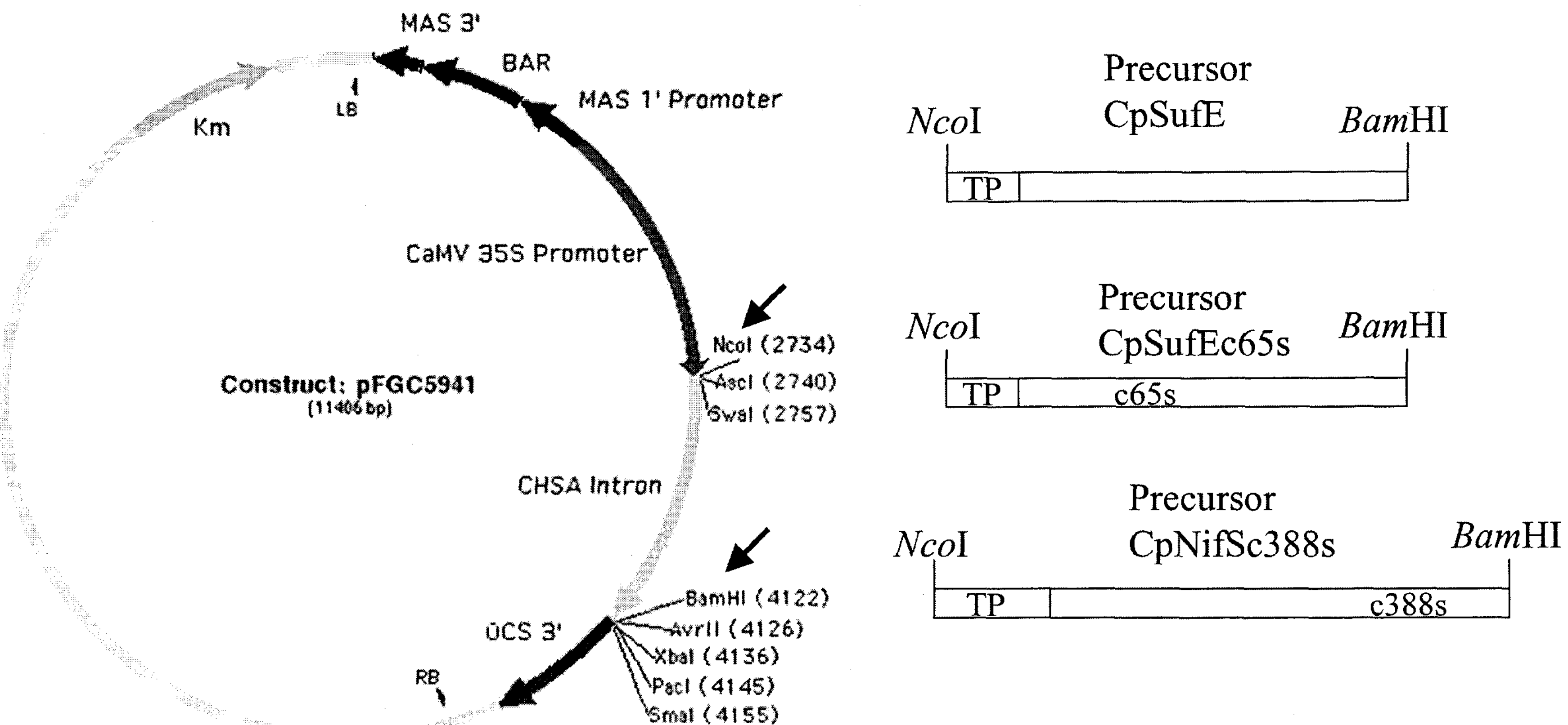

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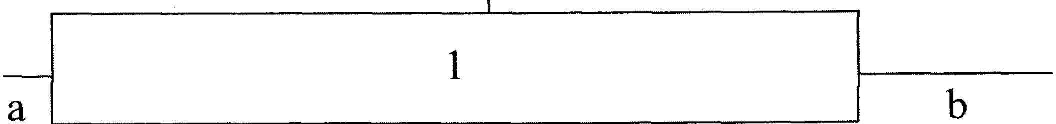
Figure 7

A



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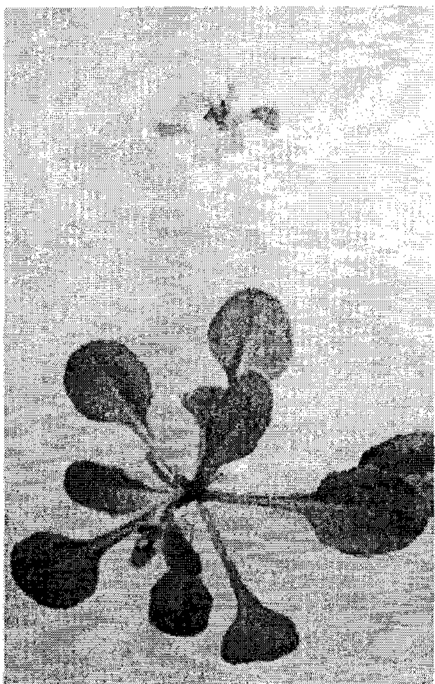

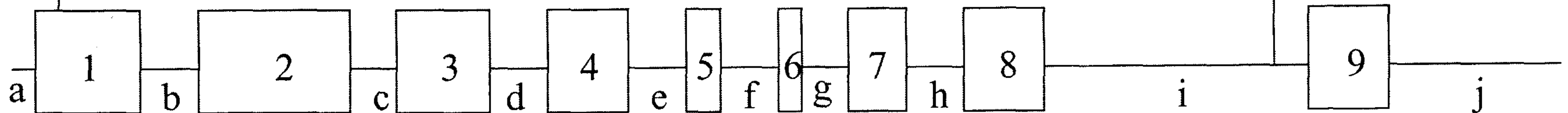

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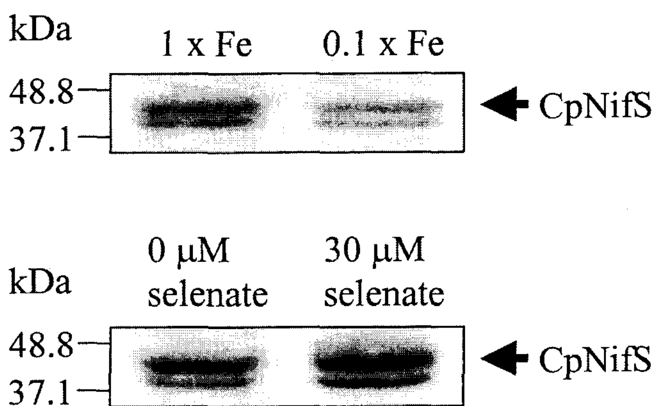
Appendix 1. Expression analysis (as repeats)



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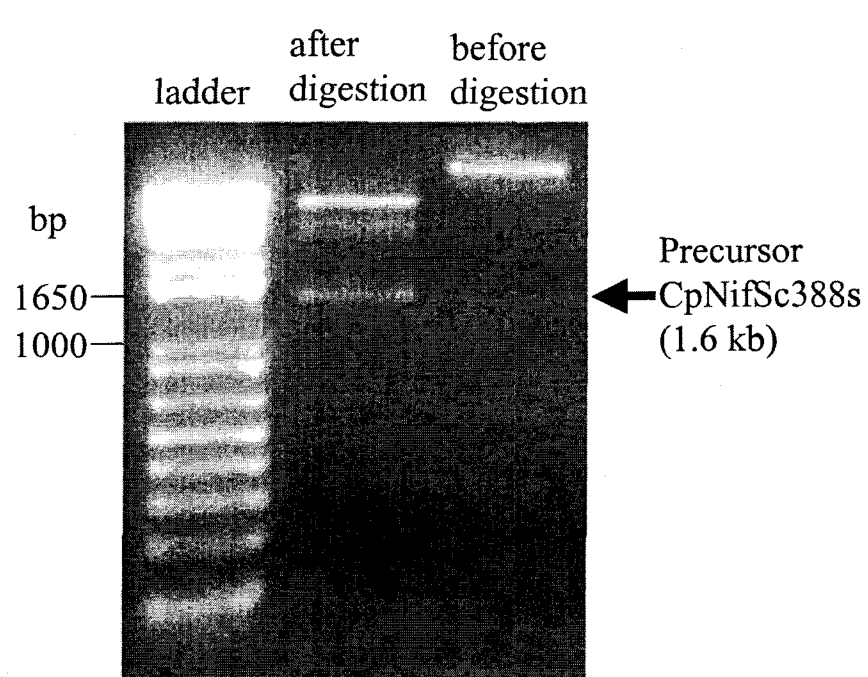
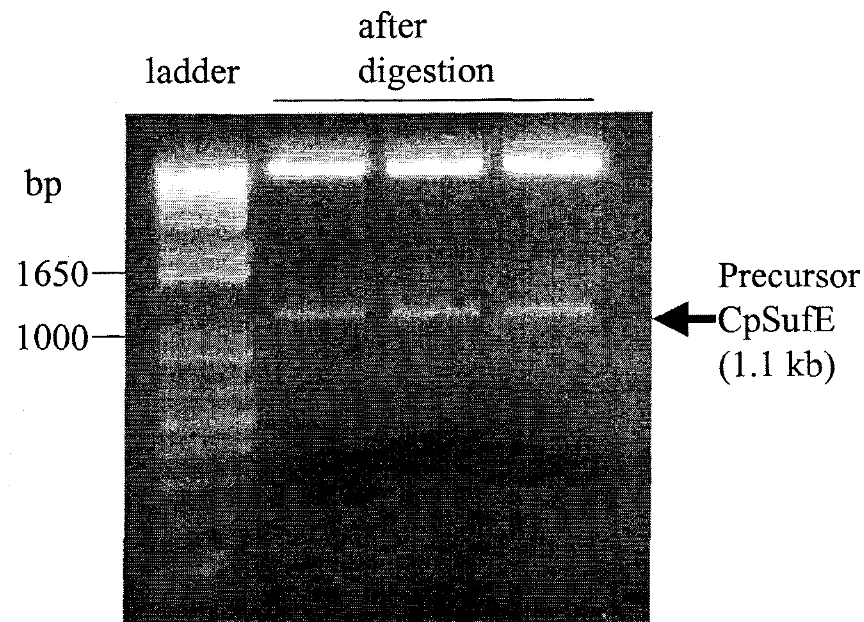
Appendix 2. DNA gels of NcoI/BamHI digestion to CpNifS/CpSufE overexpression constructs



Precursor CpNifSc388s in pFGC



Precursor CpSufE in pFGC (similar results were obtained for CpSufEc65s.)

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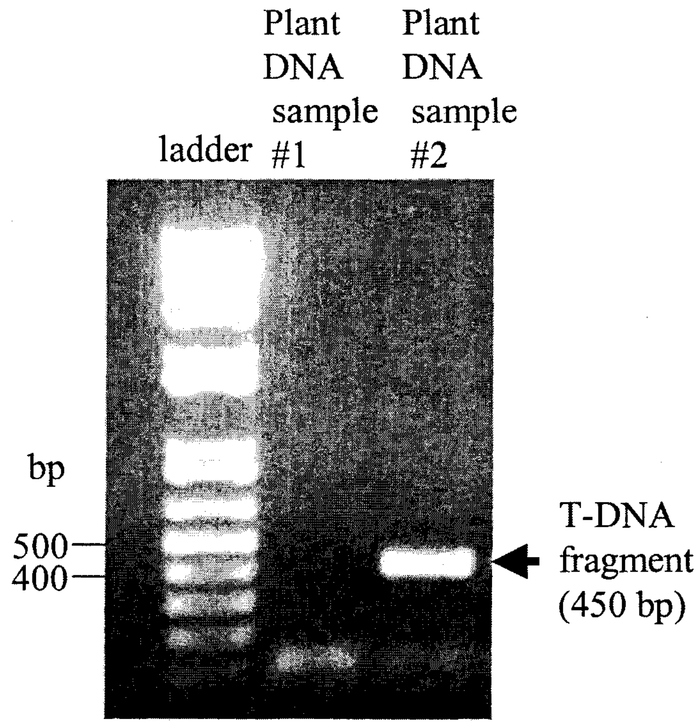
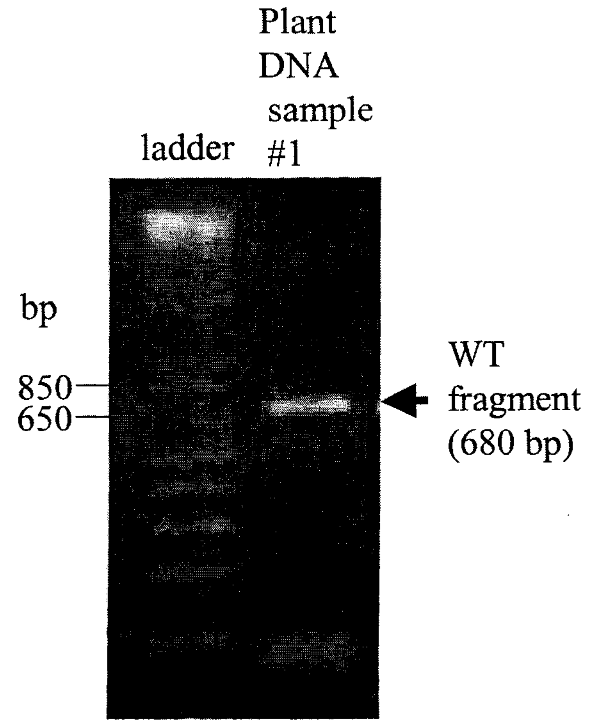
Appendix 3. DNA gels for CpSufE knockout screen



PCR for T-DNA



PCR for WT DNA

(Primer pairs and PCR program used, refer to Materials and Methods in text)

Chapter 6

Summarizing discussion

Iron-sulfur ([Fe-S]) clusters are required as prosthetic groups for a variety of chloroplast proteins, involved in photosynthetic electron transfer, nitrogen assimilation, sulfur assimilation, chlorophyll transformation and chloroplast protein import (see Chapter 1). Because it supplies [Fe-S] clusters for these essential processes, the plastidic [Fe-S] biogenesis machinery must be essential for normal chloroplast function. Also, as this machinery directly consumes iron and sulfur for cluster synthesis, it likely affects the homeostasis of iron and sulfur in plastids. This is significant, considering that iron is a limiting micronutrient for plant growth and that the [Fe-S] cluster represents a major fate of plastidic iron. Since sulfur (and selenium) assimilation largely depends on [Fe-S] proteins, this machinery likely also affects sulfur homeostasis and plant selenium metabolism.

The [Fe-S] biosynthesis machinery remained unknown until the identification of AtCpNifS, a NifS-like protein in chloroplasts (Pilon-Smits et al. 2002, Leon et al. 2002). CpNifS is a dimeric PLP-containing protein and a cysteine desulfurase (cysD) as well as a selenocysteine lyase (SL), most similar to SufS of all *E.coli* NifS-like proteins. Despite cysD activity of CpNifS being 300-fold lower than SL activity, we hypothesized that CpNifS is the cysD that provides sulfur for [Fe-S] cluster synthesis.

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This hypothesis was examined in Chapter 2. We developed an in-vitro reconstitution assay to measure [Fe-S] cluster formation by purified CpNifS or chloroplast stroma. In this assay, purified CpNifS was sufficient to catalyze the [Fe-S] cluster assembly in ferredoxin. Its reconstitution activity could be further stimulated by PLP and pyruvate, typical features described for cysteine desulfurases (Mihara et al. 2000). The chloroplast stroma also showed activity on [Fe-S] cluster reconstitution in apo-ferredoxin. The CpNifS in stroma could be removed by an affinity column containing anti-CpNifS antibodies. The resulting CpNifS-depleted stroma totally lost reconstitution ability, but the addition of an equivalent amount of purified CpNifS to the depleted stroma was able to fully restore its reconstitution activity. These results revealed that CpNifS is required for [Fe-S] formation in chloroplast stroma. The concentration of the CpNifS in chloroplast stroma was determined to be 0.06% of total proteins. Based on the same CpNifS content, the reconstitution activity of stroma is 50-80-fold more than purified protein, indicating that factors, particularly other proteins, are activating the CpNifS-mediated [Fe-S] cluster assembly in chloroplasts.

One activating factor could be a homologue of SufE. A chloroplastic version of SufE protein is described in Chapter 3. The CpSufE-encoding gene (At4g26500) has only one exon and two flanking non-transcribed-regions (NTR). The encoded CpSufE polypeptide is composed of a chloroplast targeting sequence, a N-terminal SufE domain and a C-terminal Bol-A domain. The SufE domain is found among all SufE-like proteins. It contains a highly conserved cysteine residue, which is required for activity. The function of the Bol-A domain is unknown, and only found in chloroplastic SufE proteins of higher plants. It is likely that the Bol-A domain confers

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a specific function to SufE proteins in chloroplasts, and/or that chloroplastic SufE proteins of higher plants are closely related in evolution. The chloroplastic location of AtCpSufE was confirmed by GFP localization and chloroplast immunoblot experiments. CpSufE is expressed in all tissues but at higher levels in green parts. Immunoblotting showed that CpSufE is downregulated in dark-grown plants, indicating the CpSufE expression is light-induced and it likely plays a role in photosynthesis. The recombinant CpSufE was purified from *E.coli.* and used for biochemical assays. It can form a complex with CpNifS and activate cysD activity of CpNifS 40-fold, while slightly inhibiting its SL activity. CpSufE also changed the substrate affinity of CpNifS further favoring cysteine over selenocysteine. As a consequence, CpSufE stimulated the [Fe-S] cluster formation for apo-ferredoxin 20-fold, compared to CpNifS alone. Site-directed mutagenesis experiments proved that the CpNifS cysteine388 residue is required for its cysD activity and that the CpSufE cysteine65 residue is essential for its activating function. These cysteine residues in both proteins likely constitute a sulfur transfer pathway during desulfuration. However, the cysteine65 is not required for binding ability of CpSufE. As a consequence, the mutant $CpSufE_{Cys65Ser}$ displayed a dominant-negative effect on the stimulatory activity of WT CpSufE. Despite the observation that the cysteine388 is seemingly not required for CpNifS binding, the mutant $CpNifS_{Cys388Ser}$ did not show a dominant-negative effect on WT CpNifS in vitro, probably because the mutation reduces the binding ability of CpNifS for CpSufE.

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At least one scaffold molecule has been found in each [Fe-S] biogenesis system identified so far. Indeed, an IscA-like scaffold was identified in the plastidic [Fe-S] biogenesis machinery, and reported in Chapter 4. The CpIscA is expressed in all tissues but at a higher level in green parts where chloroplasts are abundant. Its chloroplast localization was thoroughly examined by immunoblots, in-vitro import experiments, and GFP localization experiments. In reconstitution experiments, the CpIscA was able to improve CpNifS-mediated [Fe-S] assembly ~ 2-fold. The CpIscA is homologous to $IscA^{nif}$, IscA and SufA, a group of alternative scaffolds in bacterial [Fe-S] biogenesis systems. Its scaffold function was revealed in spectrophotometric and [Fe-S] cluster delivery experiments. When purified from *E.coli*, CpIscA did not have an [Fe-S] cluster, but when incubated with a ferrous salt and cysteine in the presence of CpNifS, the CpIscA protein acquired a cluster. This CpIscA was isolated and subjected to an iron content measurement. It was found that this CpIscA contained one [2Fe-2S] cluster per dimeric polypeptide, and therefore named the dimeric holo-CpIscA. The holo-CpIscA was stable and able to deliver its [2Fe-2S] cluster to apo-ferredoxin, resulting in maturation to holo-ferredoxin. This process could take place in the presence of EDTA, a strong iron chelator, indicating that the [Fe-S] cluster delivery likely involves a direct protein-protein interaction. However, the *in vivo* role of CpIscA remains to be investigated.

In the mean time, three NifU-like proteins (Nfu1-3) were found and proposed to be scaffold molecules (Leon et al. 2003; Touraine et al. 2004; Yabe et al. 2004). The Nfu2 was able to modulate formation of an [Fe-S] cluster *in vitro*. Knockout experiments supported that it is required for biosynthesis of [4Fe-4S] clusters in PSI

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and [2Fe-2S] clusters in ferredoxin. The presence of various scaffold proteins may indicate that they are responsible for [Fe-S] cluster assembly in different developmental stages, tissues, or for different target proteins (Abdel-Ghany et al. 2005). Also, plant SufBCD proteins were reported to form a complex and have ATPase activity (Xu and Moller 2004; Xu et al. 2005), maybe fueling formation of [Fe-S] clusters, or mobilizing iron. A direct involvement of the SufBCD in [Fe-S] cluster assembly still remains to be demonstrated. A mutation of the SufC resulted in accumulation of protoporphyrin IX, a precursor of chlorophyll, probably because the chlorophyll biosynthesis pathway needs [Fe-S] proteins (Moller et al. 2001). Currently, it is known that an [Fe-S] protein is involved in chlorophyll breakdown and another is involved in transformation of chlorophyll a to chlorophyll b (see Chapter 1 and Figure 1 therein). However, it has not been shown that any [Fe-S] protein(s) is/are involved in *de novo* synthesis of chlorophyll. It could be interesting to examine whether protoporphyrin IX accumulates after mutation of other [Fe-S] biosynthetic components, e.g. CpNifS and/or CpSufE. In addition, a HCF101 (Lezhneva et al. 2004) and an APO1 (Amann et al. 2004) are likely part of the [Fe-S] biogenesis machinery in chloroplasts. A working model for plastidic [Fe-S] biogenesis was proposed, as shown in Figure 2 of Chapter 1. Several members of this machinery, CpNifS, CpSufE and CpIscA, were found to be, at least in part, present in a ~600 kDa stromal complex, tentatively named the plastidic [Fe-S] synthase complex in this thesis. The other components Nfu1-3 and SufBCD might be present in the same complex. A strategy that can be used to identify individual components from the

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complex is tandem affinity purification (Rubio et al. 2005), followed by a protein composition analysis using mass spectrometry (MS).

As mentioned above, CpSufE (At4g26500) is the cysD activator of CpNifS. To date, it is the only SufE identified and characterized in chloroplasts. However, a SufE-like domain was recently identified in a putative chloroplast protein encoded by gene At5g50210 (Loiseau et al. 2005, TAIR). The protein is named AtCpNadA in this thesis. It is composed of a N-terminal SufE domain and a C-terminal quinolinate synthetase A (NadA) domain. Judged from alignment analysis, its SufE domain contains almost all amino acids that are conserved in chloroplastic and bacterial SufE proteins, particularly the essential cysteine and surrounding regions (data not shown), suggesting a SufE activity. According to BLASTp searches, its NadA domain belongs to a super-family of 211 members that are exclusively NadA proteins involved in NAD biosynthesis. Interestingly the AtCpNadA is the only NadA-like protein in Arabidopsis. Moreover, the bacterial NadA absolutely requires a [4Fe-4S] cluster for activity (Gardner and Fridovich 1991, Cicchillo et al. 2005, Ollagnier-de-Choudens et al. 2005). Therefore, a hypothesis can be postulated as follows. NAD biosynthesis in Arabidopsis, at least in part, takes place in chloroplasts. The process includes AtCpNadA (At5g50210) protein, which requires a [4Fe-4S] cluster for activity. The AtCpNadA contains its own SufE activity, stimulating CpNifS to assemble a [4Fe-4S] cluster for itself.

In fact, another putative chloroplastic SufE homologue, encoded by gene At1g67810 has been identified by BLASTp searching. According to an alignment, it also contains almost all amino acids that are conserved in chloroplastic and bacterial

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SufE proteins, particularly the essential cysteine and surrounding regions (data not shown), again likely suggesting a SufE activity. If that is true, is its function in [Fe-S] cluster biogenesis or in synthesis of other sulfur-containing molecules? Based on the embryo-lethal phenotype of CpSufE knockout, other SufE homologues, presumably present in the same compartment, cannot complement the role of CpSufE in [Fe-S] biogenesis. Therefore, it is hypothesized that At1g67810 is involved in mobilizing sulfur for synthesis of thiamine and other sulfur-molecules. Thiamine synthesis, at least in part, is thought to take place in plastids (Belanger et al. 1995).

Taken together, the working model of chloroplastic [Fe-S] biogenesis could be modified as follows. CpNifS is the central and multiple-function protein, mobilizing sulfur or metabolizing selenium, supplying sulfur for either [Fe-S] cluster biogenesis or synthesis of other sulfur-molecules. To channel the activity of CpNifS toward one of its potential functions, a SufE-mediated regulation mechanism has evolved. CpSufE can bind to CpNifS and dramatically change its catalytic and kinetic properties (see Chapter 4). This CpSufE (At4g26500) appears to be the major isoform of SufE, delivering sulfur to CpIscA and/or other scaffolds, and assembling [Fe-S] clusters for various uses. The putative CpNadA (At5g50210) likely carries a minor SufE activity in its N-terminal domain, and interacts with CpNifS transiently to assemble its own [Fe-S] cluster for NAD synthesis. The At1g67810-encoded protein is putatively a second AtCpSufE, and may interact with CpNifS transiently to mobilize sulfur for the synthesis of thiamine and other sulfur-molecules. Together these three SufE-like proteins direct the production of elemental sulfur for general [Fe-S] clusters, the NadA [Fe-S] cluster, or thiamine. In the absence of any SufE activity, the CpNifS

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is simply a seleno-cysteine lyase. Whether it has a role as such in the plant remains to be discovered. The Bol-A domain of the CpSufE (At4g26500) may enable it to be present in the ~600 kDa complex, potentially because it offers ability to interact with other proteins in the complex. Alternatively, the Bol-A domain may regulate CpNifS activity by sequestering the SufE domain of CpSufE.

It is known that several [Fe-S] biogenesis machineries play a role in iron homeostasis. For instance, in yeast and mammalian cells, impaired mitochondrial [Fe-S] biogenesis resulted in iron overload in mitochondria (for a review see Rouault and Tong 2005). Also, the Sta1 protein is an ABC transporter of the mitochondrial [Fe-S] biosynthesis machinery in Arabidopsis, the mutation of which led to iron accumulation in mitochondria (Kushnir et al. 2001). Despite these reports, a similar iron accumulation has not been found with mutations in the plastidic machinery so far. The potential involvement of the plastidic [Fe-S] biogenesis machinery in iron homeostasis as well as in sulfur homeostasis is explored in Chapter 5. The expression analysis data showed that CpNifS and CpSufE are co-regulated *in vivo*. They were upregulated in the presence of excess iron and downregulated under iron limitation, suggesting a role of the plastidic machinery in iron homeostasis. Another putative member of the plastidic [Fe-S] biosynthesis machinery, SufB, may also be involved in iron homeostasis because its ATPase activity was stimulated by iron and its transcription was downregulated under iron starvation (Xu et al. 2005). The CpIscA was downregulated under sulfur starvation, potentially suggesting an involvement of the machinery via scaffold molecules in sulfur homeostasis. However, a precise role remains to be characterized until transgenic plants with altered expression of key

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components of the plastidic [Fe-S] machinery are subjected to homeostasis analysis, as was done for selenium metabolism (Van Hoewyk et al. 2005). The CpNifS and CpSufE proteins were upregulated in the presence of selenium. Conversely, CpNifS overexpression resulted in enhanced selenium accumulation and tolerance. Together these results may suggest an involvement of the [Fe-S] biogenesis machinery, particularly CpNifS, in plastidic selenium metabolism. The investigation of the role of [Fe-S] biosynthesis machinery in homeostasis of iron and sulfur is an intriguing research area. Its results can be applied in agricultural biotechnology to improve crop productivity and nutritional quality, and in phytoremediation to use engineered plants to clean up selenium from polluted sites.

References

Abdel-Ghany, S.E., Ye, H., Garifullina, G.F., Zhang, L., Pilon-Smits, E.A., and Pilon, M. (2005) Iron-sulfur cluster biogenesis in chloroplasts. Involvement of the scaffold protein CpIscA. *Plant Physiol.* 138, 161-172

Amann, K., Lezhneva, L., Wanner, G., Herrmann, R.G., and Meurer, J. (2004) Accumulation of photosystem one1, a member of a novel gene family, is required for accumulation of [4Fe-4S] cluster-containing chloroplast complexes and antenna proteins. *Plant Cell* 16, 3084-3097

Belanger, F.C., Leustek, T., Chu, B., and Kriz, A.L. (1995) Evidence for the thiamine biosynthetic pathway in higher-plant plastids and its developmental regulation. *Plant Mol. Biol.* 29, 809-821

Cicchillo, R.M., Tu, L., Stromberg, J.A., Hoffart, L.M., Krebs, C., and Booker, S.J. (2005) Escherichia coli quinolinate synthetase does indeed harbor a [4Fe-4S] cluster. *J. Am. Chem. Soc.* 127, 7310-7311

Gardner, P.R., and Fridovich, I. (1991) Quinolinate synthetase: the oxygen-sensitive site of de novo NAD(P)+ biosynthesis. *Arch. Biochem. Biophys.* 284, 106-111

Kushnir, S., Babiychuk, E., Storozhenko, S., Davey, M.W., Papenbrock, J., De Rycke, R., Engler, G., Stephan, U.W., Lange, H., Kispal, G., Lill, R., and Van Montagu, M. (2001) A mutation of the mitochondrial ABC transporter Sta1 leads to dwarfism and chlorosis in the Arabidopsis mutant starik. *Plant Cell* 13, 89-100

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Leon, S., Touraine, B., Briat, J.F., and Lobreaux, S. (2002) The AtNFS2 gene from Arabidopsis thaliana encodes a NifS-like plastidial cysteine desulphurase. *Biochem. J.* 366, 557-564

Leon, S., Touraine, B., Ribot, C., Briat, J.F., and Lobreaux, S. (2003) Iron-sulphur cluster assembly in plants: distinct NFU proteins in mitochondria and plastids from Arabidopsis thaliana. *Biochem. J.* 371, 823-830

Lezhneva, L., Amann, K., and Meurer, J. (2004) The universally conserved HCF101 protein is involved in assembly of [4Fe-4S]-cluster-containing complexes in Arabidopsis thaliana chloroplasts. *Plant J.* 37, 174-185

Loiseau, L., Ollagnier-de Choudens, S., Lascoux, D., Forest, E., Fontecave, M., and Barras, F. (2005) Analysis of the heteromeric CsdA-CsdE cysteine desulfurase, assisting [Fe-S] cluster biogenesis in Escherichia coli. *J. Biol. Chem.* 280, 26760-26769

Mihara, H., Kurihara, T., Yoshimura, T., and Esaki, N. (2000) Kinetic and mutational studies of three NifS homologs from Escherichia coli: mechanistic difference between L-cysteine desulfurase and L-selenocysteine lyase reactions. *J. Biochem.* 127, 559-567

Moller, S.G., Kunkel, T., and Chua, N.H. (2001) A plastidic ABC protein involved in intercompartmental communication of light signaling. *Genes Dev.* 15, 90-103

Ollagnier-de-Choudens, S., Loiseau, L., Sanakis, Y., Barras, F., and Fontecave, M. (2005) Quinolinate synthetase, an iron-sulfur enzyme in NAD biosynthesis. *FEBS Lett.* 579, 3737-3743

Pilon-Smits, E.A.H., Garifullina, G.F., Abdel-Ghany, S., Kato, S., Mihara, H., Hale, K.L., Burkhead, J.L., Esaki, N., Kurihara, T., and Pilon, M. (2002) Characterization of a NifS-like chloroplast protein from Arabidopsis. Implications for its role in sulfur and selenium metabolism. *Plant Physiol.* 130, 1309-1318

Rouault, T.A. and Tong, W.H. (2005) Iron-sulphur cluster biogenesis and mitochondrial iron homeostasis. *Nat. Rev. Mol. Cell. Biol.* 6, 345-351

Rubio, V., Shen, Y., Saijo, Y., Liu, Y., Gusmaroli, G., Dinesh-Kumar, S.P., Deng, X.W. (2005) An alternative tandem affinity purification strategy applied to Arabidopsis protein complex isolation. *Plant J.* 41, 767-778

Touraine, B., Boutin, J.P., Marion-Poll, A., Briat, J.F., Peltier, G., and Lobreaux, S. (2004) Nfu2: a scaffold protein required for [4Fe-4S] and ferredoxin iron-sulphur cluster assembly in Arabidopsis chloroplasts. *Plant J.* 40, 101-111

Van Hoewyk, D., Garifullina, G.F., Ackley, A.R., Abdel-Ghany, S.E., Marcus, M.A., Fakra, S., Ishiyama, K., Ioue, E., Pilon, M., Takahashi, H., and Pilon-Smits, E.A.H. (2005) Overexpression of AtCpNifS enhances selenium tolerance and accumulation in Arabidopsis. *Plant Physiol.* In press

Xu, X.M., and Moller, S.G. (2004) AtNAP7 is a plastidic SufC-like ATP-binding cassette /ATPase essential for Arabidopsis embryogenesis. *Proc. Natl. Acad. Sci. USA.* 101, 9143-9148

Xu, X.M., Adams, S., Chua, N.H., and Moller, S.G. (2005) AtNAP1 represents an atypical SufB protein in Arabidopsis plastids. *J. Biol. Chem.* 280, 6648-6654

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Yabe, T., Morimoto, K., Kikuchi, S., Nishio, K., Terashima, I., and Nakai, M. (2004) The Arabidopsis chloroplastic NifU-like protein CnfU, which can act as an iron-sulfur cluster scaffold protein, is required for biogenesis of ferredoxin and photosystem I. *Plant Cell* 16, 993-1007

Ye, H., Garifullina, G.F., Abdel-Ghany, S.E., Zhang, L., Pilon-Smits, E.A., and Pilon, M. (2005) The chloroplast NifS-like protein of Arabidopsis thaliana is required for iron- sulfur cluster formation in ferredoxin. *Planta* 220, 602-608

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