BIOGENESIS OF IRON-SULFUR CLUSTER PROTEINS IN PLASTIDS

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SUMMARY

Iron-sulfur (Fe-S) clusters are co-factors of proteins that perform a number of biological roles, including electron transfer, redox and non-redox catalysis, regulation of gene expression, and as sensors within all living organisms, prokaryotes and eukaryotes. These clusters are thought to be among the oldest structures found in biological cells. In chloroplasts, Fe-S clusters play a key role in photosynthetic electron transport as well as nitrogen and sulfur assimilation. The capacity of the Fe atom in Fe-S clusters to take up an electron reversibly provides the required electron carrier capacity in these pathways. Iron and sulfur limitation both affect plant primary production and growth. It has long been known that iron deficiency leads to defects in photosynthesis and bleaching in young leaves, phenomena that are closely linked to a defect in chloroplastic photosystem-I (PSI) accumulation, a major Fe-S containing protein complex in plants. Although the functional importance of Fe-S cluster proteins is evident and isolated chloroplasts have been shown to be able to synthesize their own Fe-S clusters,

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much is yet to be learned about the biosynthesis of Fe-S proteins in plastids. The recent discovery of a NifS-like protein in plastids has hinted to the existence of an assembly machinery related to bacterial Fe-S assembly systems. This chapter aims to summarize what we presently know about the assembly of Fe-S clusters in plants with an emphasis on green plastids.

INTRODUCTION: IRON-SULFUR CLUSTERS, FUNCTIONS, EVOLUTION, AND FORMATION

Many Fe-S clusters are redox active due to the capacity of Fe to take up an electron reversibly. This property is used, for instance, in components of the photosynthetic electron transport chain and in the respiratory electron transport chain of mitochondria and bacteria. Next to these electron transport roles in energy-transducing systems, Fe-S proteins can have redox roles in enzymes, for instance, those involved in nitrogen and sulfur reduction. Fe-S clusters in enzymes can also have a catalytic role other than redox activity, for example, in aconitase. Finally, some Fe-S proteins function in the regulation of gene expression, and as sensors for oxygen and Fe status within all living organisms, prokaryotes, and eukaryotes (1).

The Fe in Fe-S clusters is mostly found as Fe^{3+}, with the possibility for specific Fe atoms to be reduced to Fe^{2+}. Fe-S clusters contain S as sulfide (S^{2-}) (1). In Fe-S proteins, the inorganic and acid labile S of the cluster is typically bonded with iron and it is iron that is chelated directly to the protein side-chain residues. With these general rules for the architecture, many types of cluster are possible in biological systems. Fe-S clusters can differ in the number of Fe and S atoms and the way in which they are chelated to a protein, properties that in turn can affect cluster redox potential and biological function. Furthermore, some clusters contain additional metal ions such as Ni and Mo. The most common types of clusters are 4Fe-4S clusters and the 2Fe-2S clusters. Typically, Fe-S clusters are chelated to the protein by cysteines, but other residues may contribute to chelation of the cluster. For instance, the 2Fe-2S cluster of ferredoxin-type proteins is chelated by four cysteines, with the thiol S of the protein bonding to the Fe atoms, whereas, in the Rieske-type proteins, the 2Fe-2S cluster is coordinated to two protein cysteines and two histidines (2).

The Fe-S clusters are thought to be among the oldest structures found in biological cells (3). Indeed, Fe and S may have been abundantly available in the environment in which life first evolved and the conditions at that time probably favored spontaneous Fe-S cluster formation. This availability as well as the utility of Fe-S clusters in catalysis and electron transfer may have contributed to an early "addiction" of life to Fe, particularly as Fe-S (3). Somewhat ironically, oxygenic photosynthesis, made possible by the use of Fe in electron transport systems, greatly reduced the availability of Fe due to reactivity of oxygen to Fe and the insolubility of the resulting iron oxides. Thus, present-day organisms are in fierce competition for limited available iron, despite the abundance of Fe in the earth's outer crust. Sulfur, perhaps available abundantly in reduced form in the early atmosphere, may have gradually become oxidized and is now available to plants mostly as sulfate.

Although 2Fe-2S and 4Fe-4S clusters have been assembled *in vitro* in some model proteins with ferrous iron and sulfide, it is now clear that the process is not spontaneous *in vivo* and Fe-S assembly proteins have been shown to be required for the biological formation of these [for reviews, see (4, 5)]. We aim to describe what is presently known about the Fe-S cluster assembly pathways in plants, particularly in chloroplasts.

Fe-S Proteins in Plastids

Fe-S clusters are essential components for photosynthesis, the process unique for plants and algae that drives life on earth. The Fe in Fe-S clusters plays a pivotal role in electron transfer from water to NADPH, which is used to reduce CO_2 to form sugars (6), as well as for N and S reduction and assimilation, for example, by nitrite reductase and sulfite reductase (7, 8).

The photosynthetic electron transport chain contains three major complexes, photosystem-II (PS-II), the cytochrome b_6/f complex, and PSI. In addition, the NDH complex, which is similar to the mitochondrial NADH reductase, functions in cyclic electron transport (9). Iron is present in all of these complexes, and as such Fe is the most important redox-active metal ion for photosynthetic electron transport, both quantitatively and qualitatively (10). Iron in PS-II is present in heme and non-heme iron. In the cytochrome b_6/f complex, Fe is present as heme and as 2Fe-2S Rieske-type clusters. PSI contains three 4Fe-4S clusters (11) and the electron carrier ferredoxin (Fd) contains one 2Fe-2S cluster (6). Some other plastidic Fe-S proteins are Tic55, a Rieske-type protein of the plastid protein import machinery (2Fe-2S), ferredoxin-thioredoxin reductase (4Fe-4S), sulfite reductase (4Fe-4S), nitrite reductase (4Fe-4S), and glutamate synthase (3Fe-4S) (2, 12).

The Biogenesis of Fe-S Proteins

In eukaryotes, an Fe-S assembly machinery is present in the mitochondria. Work in yeast suggested that Fe-S cluster formation is the only essential function of mitochondria. For a review see (4). Furthermore, cytosolic Fe-S clusters depend on the mitochondrial Isc machinery involving homologues of the genes encoded by the *nif/isc* clusters of bacteria and an ABC-type transporter in the mitochondrial inner membrane, which may serve to export intermediates in Fe-S assembly (13). A similar mitochondrial machinery may be present in plants (14). Mutations in the Arabidopsis Starik gene encoding a mitochondrial protein that is a functional homologue of the yeast ABC exporter required for cytosolic Fe-S cluster formation produce plants with severe growth and developmental phenotypes (14). More recently, there is also evidence for a cytosolic Fe-S cluster formation machinery in yeast (15)

Most chloroplast proteins are nuclear encoded and synthesized with a cleavable N-terminal transit sequence required for translocation into the organelle (16). Nuclear-encoded chloroplast metallo-proteins like ferredoxin (Fd) acquire their cofactors after import into the organelle (17–20). Indeed, chloroplasts appear to have their own Fe-S biosynthetic machinery: Fe-S cluster assembly in Fd was observed in isolated chloroplasts with cysteine as the sulfur donor,

a reaction that further requires light or ATP and NADPH (21, 22). Fe-S cluster assembly into radiolabeled, freshly imported ferredoxin precursor obtained by *in vitro* translation was demonstrated using isolated intact chloroplasts (17). The reaction proceeds in the absence of cytosol (19). Together, these experiments indicate the presence of an Fe-S cluster formation machinery in chloroplasts.

Following import into the organelle, the maturation of Fe-S proteins depends on a number of processes, described in more detail in the following sections. Firstly, iron must be taken up and mobilized. Secondly, sulfur must be taken up, reduced, and assimilated. Finally, Fe-S clusters must be assembled from available components and inserted into apo-proteins. Because cysteine was identified as a source for Fe-S formation in chloroplasts (21, 22), a protein with cysteine desulfurase activity is likely involved in this process. CpNifS, the first characterized NifS-like protein from plants, is the only plastid protein with this activity that has been identified (23, 24) and a CpNifS-dependent machinery likely is responsible for plastid Fe-S cluster formation.

Iron Uptake and Storage in Plants

A first step required for Fe-S formation is the uptake of iron. Although Fe is abundant in the earth's crust, it is mainly present as insoluble iron-oxide in the soil which is not bioavailable. As a consequence, Fe is one of the three most limiting nutrients to plant growth (10). Plants have developed two strategies to take up iron [for reviews, see (25, 26)]. Grasses secrete phyto-siderophores that complex iron to make it soluble and available for uptake in the root by a specialized transporter (27). Other plants like *Arabidopsis thaliana* use a ferric reductase (28) to reduce Fe(III) to Fe(II), which is more soluble and can be taken up by the IRT transporter at the root surface (29, 30). Plants also make Fe more bioavailable by pumping protons into their rhizosphere using ATPases; these protons can replace Fe and other cations at negatively charged groups on the soil surface.

Much is yet to be learned about how iron is distributed throughout the plant and inside plant cells. Fe may be chelated by nicotianamine or organic acids during long-distance transport. Iron import in the mesophyll cells may involve the activity of a ferric reductase and the action of metal transporter of the NRAMP or YSL families, but the exact mechanism is not yet clear (25). It is estimated that up to 90% of the iron in green tissues is in chloroplasts. Fe(II) transport activity has been identified for chloroplast envelopes (31); however, the molecular machinery involved is not yet identified. In leaf chloroplasts much of the Fe is used in photosynthesis, particularly in PSI, whereas the remaining or excess Fe is chelated and stored by the chloroplast protein ferritin (32). The *Arabidopis* genome encodes four different plastid ferritins, which are differentially expressed. It is very likely that the Fe used for Fe-S clusters is recruited from ferritin-bound stores.

S Assimilation and Reduction

Sulfur (S) is an essential macronutrient for plants, and present at 0.1–1% of plant dry weight depending on the plant family and soil type (10). Sulfur is generally less limiting for plant growth than other macronutrients such as N or P,

but nevertheless positive responses to S fertilization have been reported from many areas in the world including most agricultural areas. Sulfur deficiency manifests itself as chlorosis of younger leaves and stunted growth (10). The role of S in molecules is very diverse; this is because S can exist in multiple oxidation states (+6, +4, 0, −2) with different chemical properties (33). Next to its role in Fe-S clusters, sulfur is an essential element for plant primary metabolism as a structural component of proteins and lipids, antioxidants, regulatory molecules, metal-binding molecules, and co-factors/co-enzymes.

The flow of S in plants can be summarized as follows. Most S is taken up as sulfate, which is first activated and then reduced to sulfite and finally sulfide, which is subsequently incorporated into cysteine. The main form of S in soils and thus the form taken up by plants is sulfate. This is the most oxidized form of S (valence state +6), and the predominant bioavailable form in most soils. The form of S present in biomolecules is mostly reduced S, although S also occurs in its oxidized form in sulfolipids and various sulfated compounds [for a review, see (34)]. Cysteine is the first organic form of S after sulfate reduction.

The assimilation of sulfate into cysteine takes place mainly in the chloroplast. On its way from the soil to the chloroplast, sulfate enters the plant by group 1 high-affinity sulfate transporters in the plasma membrane (35-37). Translocation of sulfate to the shoot by way of the xylem appears to be facilitated by sulfate transporters from groups 4, 3, and 2 in Arabidopsis roots, involved in vacuolar efflux and xylem loading (38, 39). Sulfate is taken up from the xylem into leaf mesophyll cells, perhaps by the combined action of group 2 and 3 sulfate transporters (40, 41). From the cytosol, sulfate is transported to the chloroplasts. There may be an H^+-sulfate co-transporter in the chloroplast envelope, but so far none has been identified.

Sulfate is activated by reaction with ATP to form adenosine-5-phosphosulfate (APS). This reaction is catalyzed by ATP sulfurylase. The predominant isoform of this enzyme is located in the plastids, but there is also a minor cytosolic form; the two isoforms are regulated differently (42). The further reduction of sulfite to sulfide is mediated by sulfite reductase, a plastidic enzyme (43). The six electrons needed for this step are thought to come from ferredoxin (Fd). Sulfide is incorporated into cysteine (Cys) by coupling to O-acetylserine (OAS). This reaction is mediated by the enzyme OAS thiol lyase, also called cysteine synthase; the OAS needed for this reaction is produced by serine acetyltransferase (SAT). Because only plastidic forms of APS reductase and sulfite reductase have been found, reduction of sulfate to sulfide is thought to occur exclusively in plastids. Because of the higher reducing power in the photosynthetic chloroplasts, most of sulfate reduction probably happens in chloroplasts, although non-green plastids also perform sulfate reduction. After formation, Cys is rapidly converted to other compounds in the chloroplast or other compartments. Therefore, the Cys concentration in the cell is quite low (in the micromolar range).

Much of Cys is incorporated into proteins, either in the plastids or in the cytosol. Cysteine residues in proteins often serve an important role in protein structure and function. The structural importance is due to the capacity of two Cys thiol groups to form a disulfide bond, which can contribute to protein tertiary and quaternary structure. In intracellular proteins, thiols are mostly in a reduced

state. The reducing power of these thiol groups can be used to reduce other cell components. For instance, in chloroplasts the redox capability of Cys in thioredoxin is crucial for the regulation of photosynthetic enzymes (12). The thiol group of Cys also has metal-binding properties and is responsible for the metal-binding capacity of many metal-binding proteins including Fe-S clusters but also other proteins such as metallothioneins (44) and metal transporters such as P-type ATPases of which there are eight in Arabidopsis (45).

Cysteine holds a central position in S metabolism and is used for the biosynthesis of a variety of other reduced S compounds including methionine, S-adenosylmethionine (SAM), glutathione (GSH), and phytochelatins (PCs), the coenzymes thiamine, biotin, lipoic acid, and co-enzyme-A, the molybdenum co-factor and Fe-S clusters. About 2% of the organic reduced S in the plant is present in the form of nonprotein thiols, and around 90% of this fraction is glutathione (γ-Glu-Cys-Gly, GSH). Glutathione is synthesized enzymatically in both the plastids and the cytosol (67).

Cysteine can be converted to alanine and sulfide by Cys desulfurases (CysD). These are NifS-like proteins, that is, related in structure to the NifS protein from *Azotobacter vinelandii* (46). In Arabidopsis, one NifS-like enzyme has been reported in plastids (23, 24), whereas a second form may be present in mitochondria (14). CysD enzymes function to provide reduced S for the production of Fe-S clusters (see below) as well as several coenzymes (47).

MICROBIAL IRON-SULFUR CLUSTER BIOSYNTHETIC MACHINERIES

The study of Fe-S assembly has progressed most rapidly in microbial systems and because these studies provided very useful insights into the Fe-S machinery in plastids we provide a brief overview here. Fe-S cluster assembly in microbes can be divided into three steps: mobilization of S from cysteine and Fe from cellular stores, cluster assembly, and finally insertion in apo-proteins (5).

The first Fe-S assembly machinery studied was the *nif* system of *Azotobacter vinelandii*, which is responsible for the formation of Fe-S clusters for nitrogenase, required under nitrogen fixation conditions (46). The *A. vinelandii nif* gene cluster includes a cysteine desulfurase (CysD) encoding gene, *NifS*, as well as the other genes *nifU, nifA, NifV,* and *cysE*, all thought to be involved in Fe-S cluster formation. NifS-like proteins are pyridoxal 5'-phosphate (PLP)-dependent, enzymes that produce elemental sulfur or selenium from (seleno)cysteine, leaving alanine [(48); for a review on cysteine desulfurases, see (47)]. A second NifS-like protein occurs in *A. vinelandii*, IscS, which has a housekeeping function in the formation of other cellular Fe-S proteins (49). *IscS* is present in a gene cluster that contains paralogs of some of the *nif* genes (*iscU*, similar to the N-terminus of *nifU,* and *iscA*); thus, the *nif* and *isc* clusters have a similar organization (49). The NifU- and NifA-like proteins are thought to serve a scaffold function for the Fe-S cluster during its synthesis and before its transfer to the target protein and conserved cysteines play a pivotal role in this process (50, 51). The Isc gene cluster also includes an Hsp70 and Hsp40 and a ferredoxin-type protein. Homologues of the *nif*/*isc* genes have been discovered in several

other bacteria including *E. coli* (49) and are also present in the mitochondria of eukaryotes (4). Next to IscU- and IscA-type proteins the mitochondria have an Nfu protein, which is similar to the C-terminus of NifU (4). In yeast mitochondria, the Hsp70/40 machinery is required for the utilization of Fe-S clusters assembled on IscU (52).

In *E. coli* and *Erwinia chrysanthemi,* a third gene cluster involved in Fe-S cluster formation is the Suf operon, which also includes a NifS-like cysteine desulfurase called SufS/CsdB in *E. coli*. (53). A major function of the Suf operon may be in protecting the cell from oxidative stress and iron starvation (54, 55). Figure 1 summarizes the structures of the three gene clusters implied in Fe-S formation in bacteria. A comparison of the sequences of NifS-like proteins from various organisms reveals two classes of these proteins (48). The Isc-type cysteine desulfurases fall into class I, whereas the Suf operon encoded NifS-like protein (SufS/CsdB) falls into class II, more related to enzymes implied in selenium metabolism (Figure 2). Besides a NifS-like protein the Suf operon contains SufA, SufB, SufC, SufD, and SufE. SufA is related to NifA and IscA and may have a scaffold function (56), whereas SufE was shown to activate SufS (57, 58). SufC is a nonintrinsic cytosolic member of the ABC domain transporter super-family. It forms a complex with Suf B and D, but the biochemical role of this complex is not yet clear (54, 58).

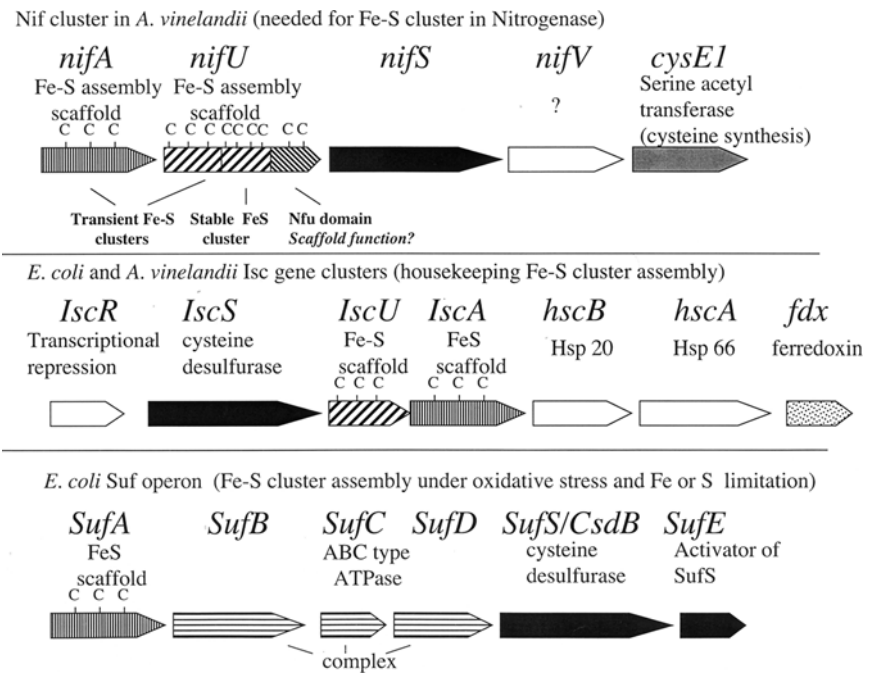

Figure 1. Overview of components of bacterial Fe-S assembly systems. Gene clusters are indicated with genes encoding proteins with similar structure indicated in a similar shading. Cysteines in scaffold proteins are indicated as c. Adapted with modification from (47).

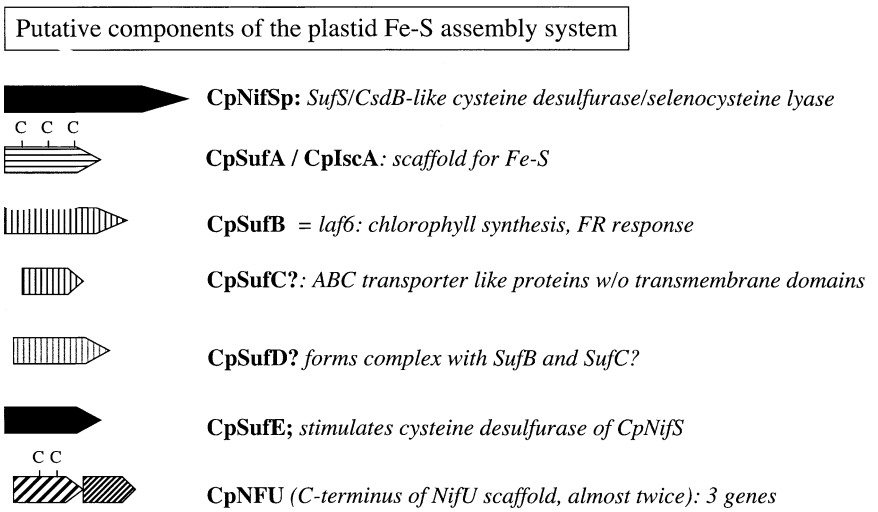

Figure 2. Schematic structure of chloroplast-localized homologues of bacterial Fe-S assembly proteins.

Chloroplasts are thought to be derived from a cyanobacterial ancestor. Even though still much is to be learned about the assembly of Fe-S clusters in cyanobacteria, it is of interest to know that the genome of non-nitrogen fixing cyanobacteria, which are perhaps the most close to plant plastids, encode homologues of IscS and Nfu (C-terminus of NifU) as well as homologues of the *E. coli* Suf operon including SufS, but proteins corresponding to IscU (or the N-terminus of NifU) are absent.

The Fe-S Assembly Machinery in Plastids

CpNifS

Inasmuch as cysteine was 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 (20). Two genes encoding NifS-like proteins have been identified in the *Arabidopsis* genome. One of the encoded proteins is present in mitochondria (14) and the other one, called CpNifS, is located in plastids (23, 24). The discovery of a NifS-like protein in plastids has prompted database searches for possible NifS-dependent protein factors that may function in Fe-S cluster assembly in chloroplasts. Putative Fe-S assembly factors with chloroplast

transit sequences are indeed encoded in the *Arabidopsis* genome (Table 1, Figure 2). CpNifS is most similar to a cyanobacterial NifS-like protein, and among the *E.coli* homologues is most similar to SufS/CsdB, a group II NifS-like protein (Figure 3). CpNifS was found to be able to use both Cys and SeCys as substrates, with a 300-fold lower cysteine desulfurase activity compared with its selenocysteine lyase activity (23).

In microbes, NifS-like proteins have also been implied to function in aspects of S metabolism other than Fe-S cluster formation, namely the biosynthesis of biotin, thiamine and molybdenum co-factor, MoCo (47). NifS-like proteins may play similar roles in plants. In bacteria and mammals, essential Se metabolism also involves NifS-like proteins, which are needed for the incorporation of Se into selenoproteins and seleno-tRNAs (47). A summary of the various possible roles of NifS-like proteins in S and Se metabolism is given in Figure 4.

It has now been shown that Se is an essential element for bacteria and animals—a requirement not yet shown for plants. On the other hand, higher Se concentrations are toxic to all organisms. Thus, organisms must prevent Se toxicity and at the same time many organisms need Se for their metabolism; NifS-like proteins may play a role in both aspects. Indeed, Arabidopsis plants that overexpress CpNifS show increased tolerance to selenate. Furthermore, transcript profiling experiments in Arabidopsis showed that a group of genes that are up-regulated in S deficiency are also up-regulated by selenate treatment, but this up-regulation is less pronounced in plants that overexpress CpNifS (Van Hoewyk et al., unpublished data). Together these results suggest that CpNifS can help reduce Se stress by avoiding Se-induced S deficiency.

The role of CpNifS in Fe-S formation was first addressed directly by Ye et al. (59). To test whether CpNifS is involved in Fe-S cluster formation for

Table 1. Putative components of a chloroplast Fe-S machinery in *Arabidopsis thaliana*.

Protein names	AGI#	Length	Proposed function	Reference
CpNifS/CpSufS	At1g08480	463	Cys-desulfurase	23, 24
CpNfu1	At4g01940	230	Scaffold	60, 61
CpNfu2	At5g49940	235	Scaffold	60, 61
CpNfu3	At4g25910	236	Scaffold	60, 61
CpSufA/CpNFA	At1g10500	180	Scaffold	Own results, Unpublished
CpSufB/Laf6	At4g04770	557	Far-red signaling?	63
CpSufC	At3g10670	338	ATPase/embryogenesis	64
CpSufD	At1g32500	475	Unclear	TAIR
CpSufE	At4g26500	371	Activator of CpNifS?	Own results, Unpublished
HCF101 (NifH-like)	At3g24430	532	4Fe-4S insertion	6

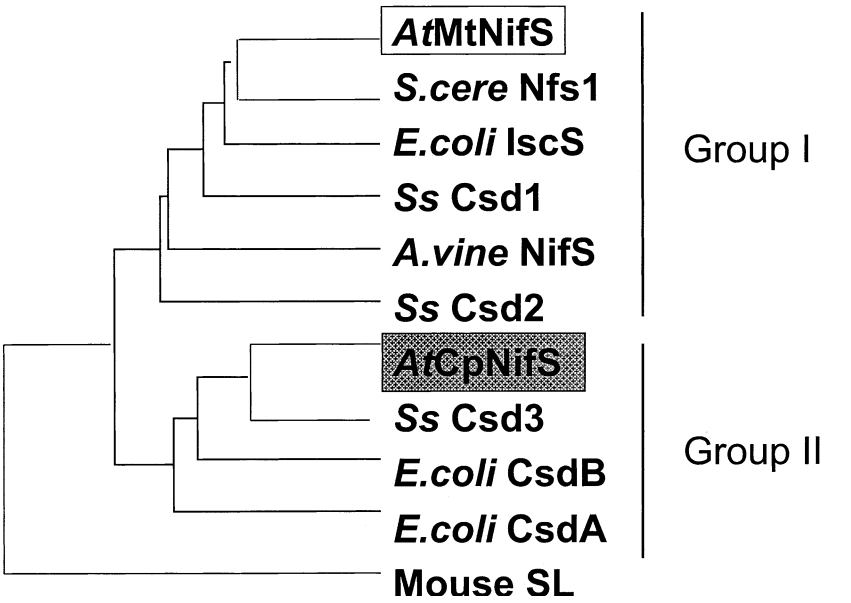

Figure 3. Grouping of NifS-like proteins based on sequence similarity. NifS-like proteins are pyridoxal-5 phosphate (PLP) dependent enzymes with both cysteine desulfurase and selenocysteine lyase activities that can be placed into two groups based on sequence similarity. In bacteria and in yeast mitochondria, cysteine desulfurases of group I with structural similarity to the NifS enzyme from *Azotobacter vinelandii* provide sulfur for Fe-S formation. Bacterial NifS-like proteins of group I such as IscS from *Escherichia coli* and *Azotobacter vinelandii* have been implied as housekeeping enzymes in Fe-S formation and are present in operons together with scaffolding proteins such as IscU and IscA . 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 SufS/CsdB in *E. coli*. AtMtNifS and AtCpNifS, the *Arabidopsis* mitochondrial and chloroplast NifS; *S. cere* NFS1, yeast mitochondrial NifS; SsCsd1, SsCsd2, and SsCsd3, synechocystis NifS-like proteins; *A. vine* NifS, *Azotobacter vinelandii* NifS; *E. coli* CsdB, SufS protein; *E. coli* CsdA, CsdA; Mouse SL, selenocysteine lyase from mouse, used as an outgroup in this phylogenetic tree (modified from 23).

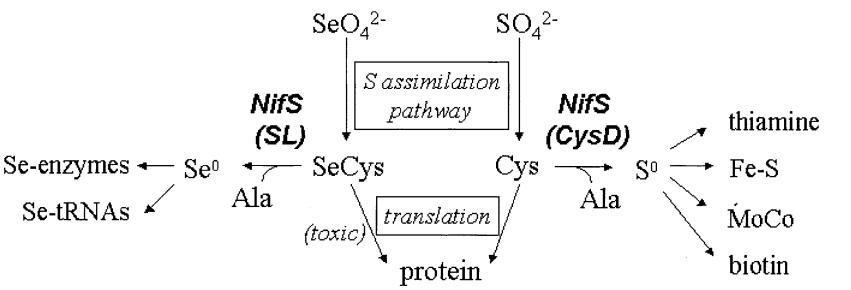

Figure 4. Possible functions of NifS-like enzymes in S and Se metabolism. Next to the function in Fe-S formation, NifS-like proteins of either group may be involved in the biosynthesis of thiamine, biotin, molybdenum cofactor, and seleno-protein and Se-tRNA synthesis.

photosynthetic proteins, an *in vitro* reconstitution assay was developed for ferredoxin. In this assay, apo-fd is 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. Purified CpNifS was active by itself in stimulating holo-fd formation in this assay. The amount of reconstituted ferredoxin was dependent on the CpNifS concentration. It was calculated that, under the assay conditions, 16 molecules of apo-Fd were reconstituted per CpNifS monomer. Thus, CpNifS has a catalytic role in iron-sulfur cluster formation in ferredoxin *in vitro*. The activity requires an intact PLP-cofactor, and CpNifS protein with a mutation of the conserved active site cysteine (Cys_{418}-Ser) is inactive, indicating that ferredoxin reconstitution involves the cysteine desulfurase activity of CpNifS.

Stromal proteins at 300 μg/mL showed activity comparable to 10 μg/mL CpNifS. Based on a quantification by means of Western blotting, we calculated that CpNifS constitutes 0.06 ± 0.02% of total stromal protein. Thus, the apparent reconstitution activity of stroma was 50-80 times more than that of pure CpNifS protein and stromal components activate CpNifS. To investigate whether Fe-S cluster reconstitution activity of stroma was dependent on CpNifS, an affinity column was used to deplete stroma of CpNifS, the removal of which was confirmed by immunoblot. Both the original stroma and the antibody-treated stroma were examined for ferredoxin reconstitution activity. The activity of the antibody-treated stroma was decreased to background levels, suggesting that the reconstitution activity of stroma was entirely dependent on CpNifS. Importantly, adding back pure CpNifS to depleted stroma to its original concentration restored the reconstitution activity. Stroma that had been treated with preimmune serum did not lose its Fe-S reconstitution activity.

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. Purified CpNifS eluted from the column in a single peak with a retention time expected for the dimer, as was found before (23). Interestingly, the CpNifS present in stroma eluted in two peaks approximately 90% eluted as a CpNifS dimer of 86 kDa, as was observed earlier using pure CpNifS. An additional, smaller amount of CpNifS eluted at a high molecular weight of ~600 kDa. This result indicates that CpNifS interacts with other proteins *in vivo* and may form a transient complex with them (59).

CpNfu as a Possible Fe-S Assembly Scaffold Protein in Plastids

Database searches (TAIR: www.arabidopsis.org) indicate that plastids do not have an IscU homologue, or a protein similar to the N-terminal domain of NifU, but several other potential members of a plastid Fe-S cluster formation machinery were identified (see Table 1 for a listing). The three *CpNFU* genes (*CpNfu* 1-3) encode chloroplast proteins that are differentially expressed but closely related in sequence to each other and similar to cyanobacterial Nfu and the C-terminus of NifU (60, 61). The domain structure of CpNfu proteins

is of interest. The three chloroplast Nfu proteins have a domain with high similarity to cyanobacterial Nfu, including the conserved cysteine that is implied in transient cluster binding. In addition, a second Nfu-like domain that lacks the cysteine residues is present at the C-terminus of the CpNfu proteins. CpNfu2 forms a transient cluster (60) that can be passed on to apo-ferredoxin *in vitro* (60, 61). Insertion mutants in one of the *CpNfu* genes (*CpNfu2*, *At5g49940*) have a dwarf phenotype and are deficient in some but not all plastid Fe-S proteins (61, 62). In the mutant lines the accumulation of both 2Fe-S and 4Fe-S proteins (PSI and sulfite reductase) is diminished and the organization of PSI is affected. Interestingly though, the KO-is viable and Fe-S protein levels were only diminished *in vitro*. Furthermore, the Rieske type 2Fe-2S of the B/F complex and the 3Fe-4S cluster of glutamate synthase were not affected (61, 62). It is possible that those clusters would require the action of any of the other two CpNfu gene products. However, the expression levels and sequence similarities of these genes may suggest that a different scaffold may be required for these substrate proteins. Because CpNfu proteins can carry a transient Fe-S cluster that can be transferred to Fd, the observed effect on Fe-S assembly in the CpNfu2 mutant is likely a direct one. However, in view of the observed effect of the CpNfu mutation on two types of clusters, it would be of interest to verify the mRNA expression levels of Fd and PSI encoding genes to rule out indirect effects of the mutations. So far, a direct link between CpNifS and CpNfu has not been established at the biochemical level, but this may only be a matter of time.

CpIscA as an Alternative Scaffold

Another potential candidate for interaction with CpNifS is CpSufA (Abdel-Ghany, Ye, Pilon-Smits and Pilon, unpublished). A T-DNA insertion line for this gene was obtained. Thus far, only plants that are heterozygous for the insertion were found. A preliminary analysis indicates that when these heterozygotes are sown on media with sucrose, one-quarter of the seedlings show a visible growth phenotype. Thus, the homozygous CpSufA knockout may be lethal in plants grown on soil, perhaps due to impaired photosynthesis. However, further analyses will be required before firm conclusions on the *in vivo* role of CpSufA can be made. CpSufA was shown to be plastidic by GFP-fusion studies. We have purified CpSufA and studied its effects on CpNifS-dependent reconstitution of Fd *in vitro*. Pre-incubation of pure CpNifS and pure CpSufA in the presence of cysteine and a ferrous iron salt was shown to give a two-fold stimulation of apo-Fd reconstitution compared with CpNifS alone. Gel filtration experiments indicated purified CpSufA is a tetramer. However, upon incubation with CpNifS, purified CpSufA acquires a transient Fe-S cluster as indicated by the absorption spectrum of CpSufA and direct measurement of Fe and it becomes a dimer. The cluster in dimeric CpSufA can subsequently be transferred to apo-Fd to form holo-Fd. Thus, CpSufA can function as an assembly scaffold for Fe-S clusters.

Other Suf-Type System Components and Hcf101

Other potential candidate proteins that may assist CpNifS in Fe-S cluster formation in plastids are the Arabidopsis homologues of SufA, B, C, D, and E and Hcf101 protein (Table 1). The putative SufB and SufC homologues are confirmed to be in the chloroplast and mutants have phenotypes that indicate a role in development (63, 64). Expression profiling indicated that the potential *SufB* gene is regulated by Fe-deficiency in Arabidopsis (65), but, so far, a link of CpSufBCD or E with CpNifS or Fe-S clusters has not been made in plants. Bacterial SufE protein is required to stimulate the low endogenous cysteine desulfurase activity of SufS/CsdB. Our laboratory has localized the plant SufE protein to the chloroplast and we subsequently labeled the protein CpSufE. CpSufE is expressed in all major tissues, like CpNIFS, and it is feasible that CpSufE and CpNifS interact. Preliminary experiments in our lab indicate that CpSufE can indeed stimulate the cysteine desulfurase activity of CpNifS (Ye et al., unpublished). It will be interesting to see what the exact physiological role of the CpSufE is. The function of the bacterial SufB, C, and D proteins is still unclear; they appear to form a complex and may be involved in providing ferrous iron, or in transferring the Fe-S cluster from the scaffold protein to the target protein. In view of the function of the bacterial Suf operon in protection from oxidative stress, Suf homologues should make suitable members of the plastidic Fe-S cluster machinery, since the chloroplast is an oxygenic compartment due to its photosynthetic oxygen production.

Another interesting putative component of the plastid Fe-S machinery is HCF101. HCF101 (high chlorophyll fluorescence 101) encodes a NifH-related P-loop ATPase that seems to be required for 4Fe-4S but not 2Fe-2S in chloroplasts (66). The mechanism of action of the protein is so far not clear.

FUTURE PROSPECTS

The mechanisms of Fe-S assembly in plastids are complex and we are far from a complete understanding of this fascinating process. Figure 5 shows a working model for the Fe-S cluster formation machinery in plastids that includes components that have been characterized biochemically. The exact role of scaffold proteins in the biosynthesis of specific Fe-S proteins requires the development of sophisticated *in vitro* systems that can measure not only 2Fe-2S insertion in ferredoxin-type proteins, but also insertion in Rieske-type proteins, 4Fe-4S and 3Fe-3S proteins. This requires novel model proteins and assays that take into account the observed need for NADPH and ATP in plastid Fe-S assembly. Furthermore, the analysis of double mutants will help reveal possible functional overlap. To assess whether effects of mutations on the accumulation of proteins is a direct effect, expression at the mRNA level should be studied.

Thus far, very little is known about the molecular details of Fe uptake in plastids. Furthermore, the regulation of Fe-storage and recruitment for Fe-S assembly is unclear. How do plants coordinate the need for Fe in photosynthesis with S metabolism and Fe uptake and mobilization? This question is not trivial since both free Fe and S are considered toxic. The newly available genetic and

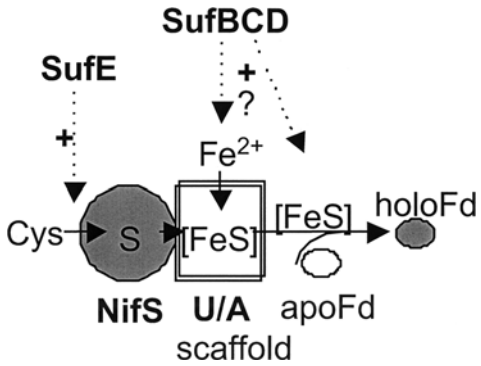

Figure 5. A working model for Fe-S formation in plastids.

genomics tools will help reveal novel elements of the Fe-S biosynthetic machinery and the regulation of the machinery as a whole in response to developmental cues, the need for photosynthesis, and nutrient status.

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