Genes from *Arabidopsis* involved in iron-sulfur cluster biogenesis

Ujwala Warek

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Biology

Dr. Brenda S. J. Winkel	Committee Chair
Dr. Dennis R. Dean	Committee Member
Dr. Charles L. Rutherford	Committee Member
Dr. Muriel Lederman	Committee Member
Dr. Craig L. Nessler	Committee Member

November 14, 2003

Blacksburg, Virginia

Keywords: *Arabidopsis,* iron-sulfur clusters

Genes from *Arabidopsis* involved in iron-sulfur cluster biogenesis

Ujwala Warek

ABSTRACT

Iron sulfur [Fe-S] proteins are essential components of many major biological processes including electron transport, respiration, photosynthesis, hormone biosynthesis, and environmental sensing. The process of [Fe-S] cluster assembly in living cells is a controlled mechanism that is highly conserved across all kingdoms. Considerable progress has been made in deciphering this mechanism in bacteria, yeast, and mammals. The key players are the NifS/IscS/SufS proteins, which act as the sulfur donor, and the NifU/IscU/SufU proteins, which serve as a scaffold that binds Fe and upon which the cluster is assembled. Additional proteins are involved in the maturation and transport of the clusters. In eukaryotes there is redundancy in the proteins involved in this mechanism and the process is compartmentalized.

Not much is known about the [Fe-S] cluster assembly mechanism in plants. In addition to the redundancy and compartmentalization seen in this machinery in eukaryotes, plants present a further challenge by offering chloroplasts as an additional site for [Fe-S] cluster assembly. The objective of this project has been to characterize *Arabidopsis* AtNFS1 and AtISU1-3, which show high homology to NifS/IscS and NifU/IscU, respectively, and are hypothesized to be key players in [Fe-S] cluster biogenesis in plants. Subcellular localization results of the AtNFS1 and AtISU1-3 proteins fused to GFP from this study are consistent with the presence of dual machinery in plants, with both mitochondria and chloroplasts as sites for [Fe-S] cluster

assembly. Furthermore, observations also showed that AtISU2 mRNA may be unstable. The results of these experiments, together with promoter analysis described in this dissertation using GUS fusions suggested that the genes encoding the AtISU scaffold proteins are regulated at the transcriptional and probably also at the posttranscriptional level.

Gene silencing experiments performed in this dissertation research using antisense and RNAi constructs indicated that these genes have the potential to impact respiration, photosynthesis, phytohormone biosynthesis, and environmental sensing, diverse processes that rely on [Fe-S] proteins. These observations, together with previous *in vitro* evidence that AtNFS1 and AtISU1 can participate in [Fe-S] cluster assembly, provide strong evidence that these proteins are part of two distinct cluster assembly systems that function in different subcellular locations and perhaps under different environmental conditions. Information gathered here has made it possible to begin developing a detailed model of [Fe-S] cluster biogenesis in plants.

AKNOWLEDGMENTS

I would like to thank Dr. Brenda Winkel for giving me the opportunity to work in her lab and for being such a superb mentor in every respect. I would also like to thank my committee members Dr. Dennis Dean, Dr. Muriel Lederman, Dr. Charles Rutherford and Dr. Craig Nessler for their advice. This adventure would not have taken place without the immense support of my husband Michael Warek, and our kids, Atreyu and Jiva. Thanks also to my sister Dr. Nikita Warner for the encouragement, my friend Deborah Johnson for always hearing me out, and the past and present members of the Winkel lab: Ana Frazzon, Lori Hill, Melissa Ramirez, Chris Dana, Daniel Owens, Dr. Anne Walton and Michelle Barthet for their assistance. I am also grateful to my parents Madhuri and Madhav Warty for their constant love.

LIST OF ABBREVIATIONS

CaMV35S	- Cauliflower Mosaic Virus promoter
CLSM	- Confocal Laser Scanning Microscopy
GFP	- Green Fluorescent Protein
GUS	- ß-glucuronidase
ISC	- Iron Sulfur Cluster
mGFP	- plant-optimized GFP variant
PSI	- Photosystem I
PSII	- Photosystem II
UV	- Ultraviolet

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1.

CHAPTER 2.

Chapter 3.

CHAPTER 4.

LIST OF TABLES

CHAPTER 1.

CHAPTER 2.

CHAPTER 3.

CHAPTER 1

LITERATURE REVIEW

Introduction

Iron-sulfur [Fe-S] clusters are small inorganic molecules that are among the most ancient and ubiquitous prosthetic groups in nature. The ability of these molecules to undergo reversible oxidation and reduction appears to have been exploited by all living organisms, and [Fe-S] clusters are found in many proteins that have important redox (as electron carriers), catalytic (in substrate binding and catalysis), or regulatory (environmental sensing) properties. [Fe-S] clusters form an important component in an array of essential processes including photosynthesis, respiration, and nitrogen fixation. Most [Fe-S] cluster-containing holoproteins (the functional form) can be reconstituted *in vitro* from the apoform. In addition, protein-bound [Fe-S] clusters can form spontaneously in the presence of inorganic sulfide and iron under anaerobic conditions *in vitro* (Merchant and Dreyfuss, 1998). However, because free Fe and S are toxic to the cell, it is unlikely that these metals exist in appreciable free quantities in the cell and there is now good evidence that biological [Fe-S] cluster formation occurs via a controlled process mediated by a specific set of proteins.

The mechanism of [Fe-S] cluster assembly was initially discovered in 1993 in the facultative anaerobic, diazotrophic (i.e. nitrogen-fixing) bacterium, *Azotobacter vinelandii* (Zheng et al., 1993; Fu et al., 1994). This discovery opened a dynamic new area of research in the characterization of [Fe-S] cluster biogenesis. Considerable progress has been made in understanding this process, particularly in *A. vinelandii*, *Escherichia coli*, yeast, and mammals (reviewed in, Lill and Kispal, 2000; Frazzon et al., 2002; Mihara and Esaki, 2002). Identification of the major players in [Fe-S] cluster assembly has been facilitated by the fact that the corresponding genes are organized as operons in

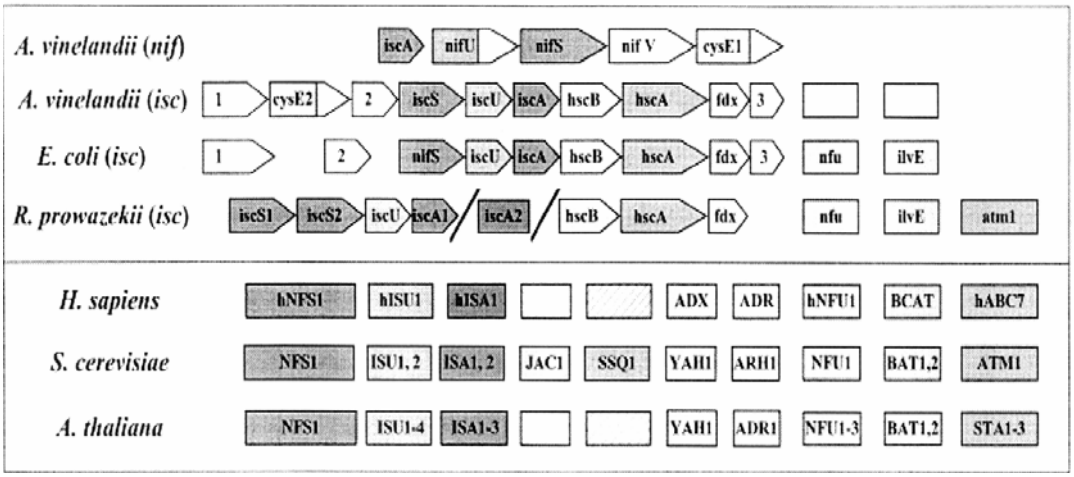
eubacteria (Figure 1). Specific proteins have been shown to control [Fe-S] cluster formation in bacterial and yeast models. In addition, there is evidence that this process is compartmentalized in eukaryotes (Lill and Kispal, 2000; Tong and Rouault, 2000; Léon et al., 2003). However, the details of how [Fe-S] clusters are assembled and inserted into target proteins are only just beginning to emerge.

In particular, not much is known about the assembly mechanism of [Fe-S] clusters in plants. The *Arabidopsis* genome contains homologs to almost all known components of the [Fe-S] cluster assembly mechanism. Small gene families encode most of the proteins and many are represented in the publicly-available gene knockout collections. *Arabidopsis* thus provides an ideal opportunity for investigating [Fe-S] cluster biogenesis in plants using well-established genetic and biochemical tools together with emerging technologies in genomics and proteomics.

The *nif and isc* operons in *Azotobacter vinelandii*

The study of the mechanism of [Fe-S] cluster assembly for the nitrogenase enzyme of *A. vinelandii* (Jacobson et al., 1989; Jacobson, 1989a) identified a requirement for two key proteins, encoded by the *nif* operon, NifS and NifU, as well as a number of accessory proteins including proteins encoded by *nifV* and *nifM*, which are adjacent to *nifU* and *nifS* in the *nif*-cluster and form a distinct transcription unit (Jacobson, 1989a). These genes, which do not form part of the structural components of the nitrogenase, were shown to be necessary for full nitrogenase activity.

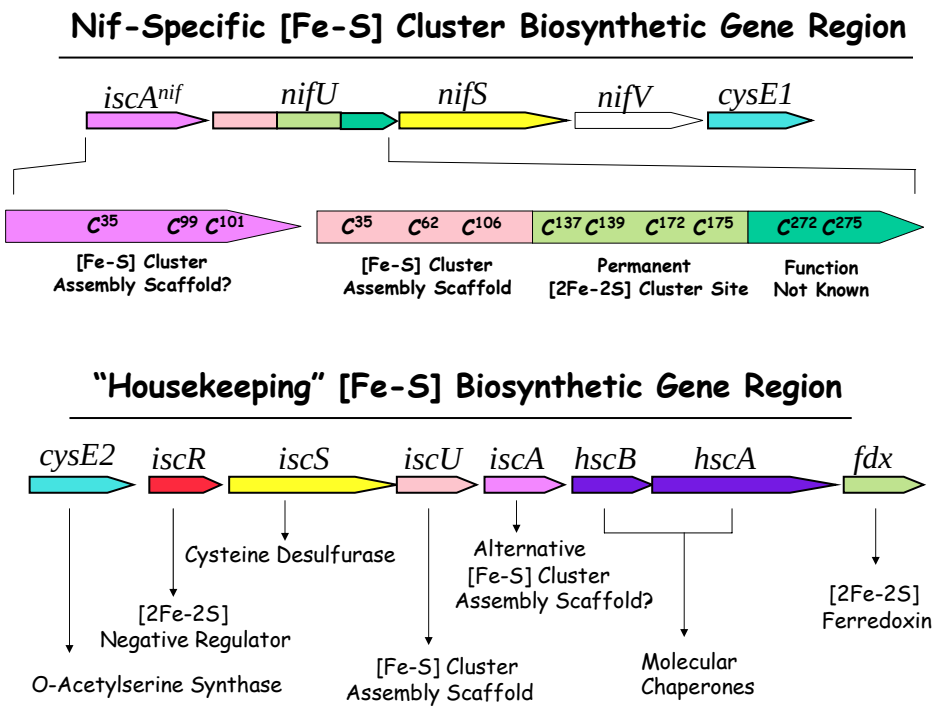
Figure 1. Components of the [Fe-S] cluster biosynthesis machinery in bacteria and eukaryotes. (Upper panel) comparative organization of a part of the nitrogen-fixation (*nif*) gene cluster from *Azotobacter vinelandii* and the *iscSUA* gene cluster from *A. vinelandii, Escherichia coli,* and *Rickettsia prowazekii*. Additional components that are involved in [Fe-S] biosynthesis in bacteria, but which are not members of the *nif* or *isc* gene clusters, are displayed by rectangles. (Lower panel) components involved in [Fe-S] cluster assembly in human, unicellular eukaryotes (*Saccharomyces cerevisiae)* and plants (*Arabidopsis thaliana*). Hatched boxes indicate components that are assumed to exist, but have not so far been identified (Figure adapted from Mühlenhoff and Lill, 2000).



A second key observation was that, although the *nifS*- and *nifU*- deficient mutants of *A. vinelandii* were strongly inhibited in the ability to form active nitrogenase, these cells were able to synthesize other [Fe-S] proteins (Jacobson et al., 1989). This led to the discovery of a second set of proteins encoded within the *A. vinelandii* genome that are responsible for [Fe-S] cluster assembly in processes other than nitrogen fixation (Zheng et al., 1998). These proteins were designated IscS (homologous to NifS) and IscU (homologous to NifU) form part of the (iron-sulfur cluster) *isc* operon (Figure 2). Inactivation of *iscS* or *iscU* from *A. vinelandii is* not possible, indicating that the isc proteins are involved in the general "housekeeping" pathway of cellular [Fe-S] cluster assembly and are essential for growth in this organism (Zheng et al., 1998). The homologous iscS, iscA, hscA, and hscB proteins were also shown to be important for [Fe-S] cluster biosynthesis in *E. coli* (Takahashi and Nakamura, 1999) and overexpression of the entire *isc* cluster has been shown to increase the yield of recombinant [Fe-S] holoproteins in this bacterium (Nakamura, 1999).

The *isc* genes are highly conserved and ubiquitous, with homologs present in all organisms examined to date (Table I), including other Eubacteria, Archaea, yeast, plants, and humans (Lill and Kispal, 2000; Mihara and Esaki, 2002). However, a complete set of isc components is not always present in these organisms. In bacterial genomes sequenced so far, with a few exceptions, these genes are arranged as in *E. coli*. Interestingly, the obligatory intracellular bacterium, *Rickettsia prowazekii*, believed to be the closest present-day relative to the ancestor of mitochondria (Andersson, 1998) contains a split version of the *isc* operon (Mühlenhoff and Lill, 2000).

Figure 2. Organization of *A. vinelandii* Nif and Isc genes involved in [Fe-S] cluster biogenesis. C stands for conserved cysteines. Figure courtesy of Dennis Dean.



The *suf* operon in *Escherichia coli*

Another system, designated *suf* for its role in sulfur mobilization has been found to play a role in [Fe-S] cluster assembly in pseudorevertants of an *isc* mutant strain of *E. coli* (Takahashi and Tokumoto, 2002; Outten et al., 2003). This system is specified by the *E. coli sufABCDSE* operon and genes with close homology to the various components of the *suf* operon are found across a wide range of Eubacteria and Archaea and in the plastids of some parasites. However, while some of these organisms carry both the *isc* and *suf* operons in their genomes, others, including *A. vinelandii*, completely lack the SUF machinery (Ellis et al., 2001; Mihara and Esaki, 2002; Takahashi and Tokumoto, 2002). In an *E. coli* mutant in which the entire *isc* operon has been deleted, the activity of [Fe-S] proteins is only 2-10% of that seen in wildtype cells (Takahashi and Tokumoto, 2002). This is presumably due to the presence of the *suf* genes, as supported by the fact that overexpression of the *suf* operon restores the growth phenotype and activity of [Fe-S] proteins in mutant cells lacking all components of the isc machinery. Furthermore, it was not possible to disrupt both *isc* and *suf* operons (Takahashi and Tokumoto, 2002). The suf system is simple because it lacks equivalents to the ferredoxin protein, Fdx, and the chaperones, HscA and HscB present in the isc system. In addition, it contains genes that are not present in the isc operon thus presenting a novel system for [Fe-S] cluster assembly.

The SufS (CsdB) protein, which is similar to NifS and, as discussed below, to *Arabidopsis* AtNFS2, was characterized and shown to have a higher affinity to seleno-cysteine than cysteine (Mihara et al., 1999; Lima, 2002; Mihara and Esaki, 2002). New data shows that SufS is in fact a two component cysteine desulfurase enzyme that

increases its affinity to cysteine up to 50-fold in the presence of SufE and the SufBCD complex (Outten et al., 2003). This sulfur transfer mechanism has been suggested to be important for limiting sulfide release during oxidative stress (Loiseau et al., 2003; Outten et al., 2003). SufA is more similar to IscA and SufB, C, and D are most likely to be components of an ABC transporter machinery, while SufE remains to be characterized. In *E. coli*, the *suf* operon is required for the stability of certain [Fe-S] proteins and is known to be regulated by oxidative stress and the iron status of the cell (Muller et al., 1998; Patzer and Hantke, 1999). A major effect of oxidative stress is damage to [Fe-S] clusters, providing strong support for the role of the SUF machinery in [Fe-S] cluster assembly during repair mechanisms.

Structure and function of the NifS/IscS/SufS-like proteins

Of all the components of the [Fe-S] cluster biogenesis machinery, NifS and IscS are the best characterized. NifS and IscS are both homo-dimeric, pyridoxal-5'-phosphate (PLP)-dependant enzymes that can catalyze the release of elemental sulfur from L-cysteine to provide the inorganic sulfur necessary for [Fe-S] cluster formation (Zheng et al., 1993). Inhibitors of PLP-containing enzymes can inhibit NifS, indicating the involvement of this cofactor and a nucleophilic cysteine in the enzymatic activity of the protein (Zheng et al., 1994). In addition, thiol-alkylating agents strongly inhibit NifS. Using site-directed mutagenesis, a specific cysteine residue of NifS was shown to be essential for enzymatic activity (Zheng et al., 1994). The three-dimensional structures of IscS from the thermophillic bacterium, *Thermotoga maritima* (Kaiser, 2000), and its homologs, the selenocysteine-lyase *from E. coli*, CsdB (Fujii, 2000), and cystine C-S-

lyase from *Synechocystis* sp. PCC 6803 (Clausen, 2000), have been solved. All of the Nif-S like proteins have two domains that are connected by two α-helices. The smaller C-terminal domain binds the reactive cysteine residue while the larger domain is linked to the PLP cofactor, which is covalently attached to a lysine residue. In contrast to this detailed understanding of the desulfurase activity of NifS, the mechanism of sulfur release from NifS during [Fe-S] cluster assembly is not yet understood. The second class of NifS/IscS-like proteins includes SufS and Suf-like proteins which have a higher affinity to selenocysteine than cysteine. A third class of NifS/IscS-like proteins is defined by the C-DES cysteine desulfurases of the cyanobacteria, *Synechocystis* PCC 6803 and *Thermosynechococcus elongates*, that has been shown to assemble the [Fe-S] cluster of ferredoxin *in vitro* (Leibrecht and Kessler, 1997; Nakamura et al., 2002).

Structure and function of NifU and IscU

Similar to NifS, NifU has also been shown to be essential for full nitrogenase activity in *A. vinelandii* (Jacobson et al., 1989; Jacobson, 1989a). NifU is an Fe-binding protein that serves as a scaffold for intermediate cluster assembly (Yuvaniyama et al., 2000). The NifU protein exists as a homodimer and carries two identical permanent [2Fe-2S] clusters (Fu et al., 1994), which give it unusual spectroscopic properties, probably resulting from the distinct arrangement of coordinating cysteine residues in the [2Fe-2S] clusters.

The NifU protein has a modular structure composed of three sections (Agar et al., 2000c). The N-terminal domain acts as a scaffold during the assembly process and shows sequence identity with the IscU and ISU proteins. IscU proteins from bacteria

that are unable to fix nitrogen (non-diazotrophic bacteria) are considerably smaller and are among the most highly-conserved proteins in nature (Hwang, 1996). The IscU protein contains three conserved cysteine residues that constitute a labile mononuclear iron-binding site (Agar et al., 2000b). Site-directed mutagenesis of *S. cerevisiae* Isu1p which shares homology with the N-terminal domain of NifU and *A. vinelandii* NifU showed that all three cysteine residues are necessary for function (Garland, 1999; Agar et al., 2000b). The central domain of the NifU protein contains a permanent [Fe-S] cluster and is involved in transfer of the transient cluster. There is no apparent homolog in non-diazotrophic organisms for this central domain. The function of the C-terminal domain of NifU, which contains a CXXC motif, is not yet known. Substitution of the cysteine residues with alanine did not have any deleterious effects on the diazotrophic growth of *A. vinelandii* (Agar et al., 2000c). Proteins designated Nfu in cyanobacteria, Nfu1p in yeast and mammals, and as discussed below, AtNFU1-5 in *Arabidopsis*, show homology to the C-terminal portion of the diazotrophic NifU (Schilke et al., 1999; Léon et al., 2003).

The existence of additional [Fe-S] biogenesis mechanisms other than those encoded by the *nif/isc/suf* operons is suggested by organisms which do not contain a complete set of these operons. For example, the cyanobacterium *Synechocystis* contains no homologs of IscU, but three IscS-like homologs all of which have the ability to incorporate [Fe-S] clusters into ferredoxins (Leibrecht and Kessler, 1997; Jaschkowitz and Seidler, 2000; Kato et al., 2000). Although *Synechocystis* PCC 6803 lacks a gene with homology to *iscU*, it has a gene encoding a protein (Nfu) similar only to the C-terminal part of NifU. This protein is proposed to contain a [2Fe-2S] cluster that can be

delivered to ferredoxin without the aid of any other factors, thus functioning as a scaffold for [Fe-S] cluster assembly (Nakai et al., 1998). Similarly, *Rhodobacter capsulatus* produces a protein that is similar only to the C-terminus of *A. vinelandii* NifU (Masepohl et al., 1993). This is interesting considering the fact that the function of the C-terminal domain of *A. vinelandii* NifU is unknown. Even more intriguing is that some Archaea do not possess any apparent homologs of the isc machinery although they do contain [Fe-S] proteins.

Other genes of the *isc* operon

As illustrated in Figure 2, in addition to IscS and IscU, the *isc* operon encodes two heat-shock proteins (hsc) and three other proteins, IscA, IscR, and ferredoxin (Fdx). The *hscB* and *hscA* genes show high homology to DNAJ/Hsp40 and DNAK/Hsp70 respectively, and encode proteins that are likely to serve as molecular chaperones that specifically assist in the maturation of [Fe-S] proteins (Hoff et al., 2000). Although the hsc proteins are required for the full activation of [Fe-S] proteins in *A. vinelandii* and *E. coli,* their specific functions have not yet been determined (Zheng et al., 1998; Hoff et al., 2000; Tokumoto and Takahashi, 2001). Both proteins interact with IscU and a complex between HscA and IscA has also been detected, leading to speculation that they are involved in optimizing conformation of the cluster complex prior to transfer of the cluster from the scaffold proteins to the target apoproteins (Hoff et al., 2000; Tokumoto et al., 2002). However, it is interesting to note that NifU of *Synechocystis PCC6803* (SyNifU) can deliver its $[2Fe\text{-}2S]^{2+}$ cluster to apoproteins *in vitro* without any aid from other factors (Nishio and Nakai, 2000). *Thermatoga maritima* IscU has also

been shown to deliver [Fe-S] clusters to apoferredoxin without the aid of HscA and HscB (Mansy et al., 2002). IscA appears to function as an alternative scaffold for [Fe-S] cluster assembly, perhaps providing for the assembly of other types of clusters, i.e. $[2Fe\text{-}2S]^{2+}$ or $[4Fe\text{-}4S]^{2+}$, targeting different acceptor proteins, or providing activity under different physiological conditions (Krebs et al., 2001). Interestingly, iscA homologs have not been identified in any of the archaeabacterial genomes sequenced to date (Mühlenhoff and Lill, 2000). IscR was recently discovered to be involved in negative feedback transcriptional regulation of the *isc* operon in *E. coli*, providing an essential mechanism for controlling the accumulation of excess [Fe-S] clusters and deleterious formation of insoluble iron sulfides (Schwartz et al., 2001; Frazzon et al., 2002). No direct counterpart of IscR has been found in eukaryotes. Fdx is a [2Fe-2S] protein and there are some indications that it may serve as a reductant at some point during cluster assembly. Both IscU and IscA can form complexes with Fdx, suggesting that electron transfer occurs between these proteins but its exact function is not yet known (Ollagnier-de-Choudens et al., 2001; Wollenberg et al., 2003).

The mechanism of [Fe-S] assembly

The major players in [Fe-S] cluster assembly, NifS and NifU, form a complex during the catalytic formation of the [Fe-S] precursor on the NifU scaffold. The sulfur is delivered from NifS to NifU, upon which a [Fe-S] cluster is formed and then transferred to the nitrogenase component proteins. This was confirmed by biochemical analysis that showed that NifS can assemble a transiently-bound [2Fe-2S] cluster on the N-terminus of the IscU-homologous part of NifU *in vitro* (Yuvaniyama et al., 2000). Similar findings

were also made for IscU from *A. vinelandii* (Agar et al., 2000b). The mechanism of cluster assembly on NifU is not completely understood. The *in vitro* data suggests that release of the transient cluster from NifU occurs via a reduction step that may involve the permanent [Fe-S] clusters on NifU. Permanent [Fe-S] clusters were shown to play an essential role in the assembly of nitrogenase in *A. vinelandii* by site-directed mutagenesis of cluster-ligating cysteine residues of NifU (Agar et al., 2000a). In IscU, which lacks a permanent [Fe-S] cluster, the [2Fe-2S] ferredoxin may take over the role of the permanent [Fe-S] clusters of NifU.

The isc machinery in non-diazotrophic bacteria

Most of the analysis of the mechanism of [Fe-S] cluster biogenesis in non-diazotrophic bacteria has been carried out in *E. coli* and the cyanobacterium *Synechocystis.* The *isc* gene cluster in *E. coli* is similar to that in *A. vinelandii*, but contains two additional genes with products that bear homology to IscS (Takahashi et al., 1991; Schwartz et al., 2000). Knockout mutants of *iscS* in *E. coli* grow at only half the rate of the wild-type strain in rich medium (Lauhon and Kambampati, 2000; Schwartz et al., 2000). These mutants exhibit a growth delay when switched to minimal medium that is alleviated by the addition of isoleucine and valine. Furthermore, these mutants are auxotrophic for thiamine and nicotinic acid under aerobic conditions. A *nifS*-like mutant in *Bacillus subtilis* shows similar nicotinic acid-dependency (Sun and Setlow, 1993). Deficiency in the maturation of [Fe-S] proteins that participate in the nicotinic acid and isoleucine/valine biosynthetic pathways is believed to be the cause of these phenotypes. One important outcome of studying the NifS/IscS homologs in

various organisms has been the identification of additional roles for these proteins in processes unrelated to [Fe-S] cluster assembly. These functions include the role of IscS as a sulfur donor for the biosynthesis of thionucleosides in *E. coli* and *Salmonella enterica* (Kambampati and Lauhon, 2000; Lauhon, 2002; Nilsson et al., 2002), the thiazole moiety of thiamine in *E. coli* (Xi et al., 2001), and molybdopterin cofactor in *Aspergillus* and *Drosophila* (Amrani et al., 2000), in lipoid acid and biotin synthesis (Marquet et al., 2001) and NAD and branched-chain amino acid (isoleucine/valine) biosynthesis (Sun and Setlow, 1993), as well as in tRNA splicing in yeast (Kim et al., 1995). These findings suggest that the NifS/IscS/SufS family of proteins has a broad role in sulfur and selenium trafficking and utilization.

The isc machinery in eukaryotes

Homologs of components of the bacterial isc machinery are observed in all eukaryotes, including plants and animals, pointing to a bacterial origin and high conservation of the isc machinery. Most of the [Fe-S] proteins in eukaryotes are located in mitochondria (Lill et al., 1999). For example, in the mitochondrial respiratory chain, the first energy-coupling site contains at least four [Fe-S] proteins. Aconitase, the second enzyme in the citric acid cycle is also a [Fe-S] protein (Jordanov et al., 1992). However, mature [Fe-S] proteins are also found in chloroplasts, the cytoplasm, and the nucleus. [Fe-S] proteins found in the chloroplasts include components of the chloroplast inner envelope protein apparatus (Imsande, 1999), three enzymes of the Calvin cycle and first enzyme of the pentose phosphate pathway (Staples et al., 1996; Imsande, 1999). Cytoplasmic [Fe-S] proteins include glutamate synthase, a subunit of

sulfite reductase, isopropyl malate synthase and the mammalian iron regulatory protein 1 (IRP-1), among others, while the nucleus harbors [Fe-S] proteins such as the endonuclease Ntg2p (reviewed in Mühlenhoff and Lill, 2000). These observations raise the questions of whether all [Fe-S] clusters in eukaryotes are synthesized by a mechanism located at one site or by mechanisms located at different subcellular sites.

Most of the information on the eukaryotic isc machinery has come from investigations using *S. cerevisiae.* These studies have shown that mitochondria perform a crucial function in the biogenesis of [Fe-S] clusters in eukaryotes (Craig et al., 1999; Lill et al., 1999). Genetic and biochemical analyses have identified ten components that share significant homology to bacterial proteins involved in [Fe-S] cluster assembly. These include the functional counterparts of bacterial NifS/IscS (Nfs1p), the NifU C-terminus (Nfs1p), the NifU N-terminus (Isu1p and Isu2p), IscA (Isa1p and Isa2p), fdx (Yah1p), and two heat shock cognate proteins (Ssq1p and Jac1p). Although these proteins are all translated in the cytoplasm, they are finally located in the mitochondrion (Kaut et al., 2000; Lill and Kispal, 2000; Pelzer et al., 2000).

Eukaryotic Nfs1 proteins are highly homologous to bacterial NifS and IscS and are believed to also function as cysteine desulfurases. *E. coli* IscS expressed in yeast mitochondria could fully replace the essential Nfs1p, indicating that Nfs1p and IscS perform similar functions (Kispal et al., 1999). However, IscS and Nfs1p were not functional when expressed in the yeast cytosol, implying that the sulfan sulfur could only be provided within the mitochondria. Nfs1p was also found to mediate the uptake and intracellular distribution of iron in yeast cells (Li et al., 1999). Further, Kispal et al.,

(1999) showed that yeast cells exhibited a strong defect in the activity of [Fe-S] cluster-containing proteins when Nfs1p was depleted by regulated gene expression. These observations suggest that these proteins function in [Fe-S] cluster assembly in the yeast mitochondrion.

The Isu1 and Isu2 proteins of yeast show high homology to bacterial IscU and the N-terminus of NifU and are also believed to function as scaffold proteins. These proteins play a central and complementary role in [Fe-S] cluster assembly as deletion of one of the *isu* genes results in relatively weak growth and defects in mitochondrial [Fe-S] proteins while the deletion of both the *isu* genes leads to quantitative loss of [Fe-S] protein function within the mitochondria and is lethal to the organism (Schilke et al., 1999). Targeting bacterial IscU to mitochondria complemented these defects, implying that IscU and the two yeast Isu proteins are functional orthologs (Mühlenhoff and Lill, 2000). Like IscU, the Isu1 protein contains three conserved cysteine residues. This was determined by complementation studies using Isu1 mutants that were generated by site-directed mutagenesis at each of the cysteines and *Δisu1* strains (Garland, 1999).

Why mitochondria?

It is interesting to consider the reasons why nature has chosen mitochondria as a site for [Fe-S] cluster production. The primeval cell may have harbored two systems capable of [Fe-S] cluster assembly shortly after successful endosymbiosis. One system may have been that of the prokaryotic endosymbiont resembling the current isc machinery and the other of the archaeabacterial type in the host cytoplasm, which was then lost during evolution in some organisms such as yeast, but retained in others such

as mammals. Localization of the [Fe-S] cluster machinery in mitochondria may be advantageous due to the reducing environment provided by these organelles, as evidenced by the absence of disulfide bridges in mitochondrial proteins, and the considerably lower partial oxygen pressure compared to the cytoplasm. Both are factors that favor [Fe-S] synthesis because IscU and IscU homologs have conserved cysteines that need to be maintained in a reduced form for the activity of this protein. Further, the absence of any accumulation of apoproteins in the mitochondrial matrix confirms that apoproteins are not imported into the mitochondrion.

Biogenesis of extra-mitochondrial [Fe-S] proteins

The mechanism by which the labile [Fe-S] cluster is exported from mitochondria is not yet fully known. Export of [Fe-S] clusters for maturation of extra-mitochondrial proteins has been proposed to be mediated by the inner membrane ABC transporter, Atm1p, and the sulfhydryl oxidase, Erv1p, of the mitochondrial intermembrane space and requires a mitochondrial membrane potential (Lange et al., 2001). Genetic analysis has shown that these genes are essential for the survival of yeast. Atm1p is apparently conserved within the eukaryotic kingdom, and homologues of this gene in human (*hABC7*) and plants (*Arabidopsis AtSTA1*) can complement the function of this transporter, leading to almost normal biosynthesis of cytoplasmic [Fe-S] proteins in yeast ΔAtm1p, mutants (Csere et al., 1998; Bekri et al., 2000b; Kushnir et al., 2001). In humans, mutations in hABC7 have been associated with an increase in mitochondrial iron levels, as was seen in yeast (Schilke et al., 1999), and linked to one form of an iron storage disease, an X-linked sideroblastic anemia and ataxia (Allikmets et al., 1999;

Bekri et al., 2000a). In contrast, *Arabidopsis* defective in the ABC transporter, AtSTA1, exhibit dwarfism and chlorosis, but no significant increase in mitochondrial iron levels (Kushnir et al., 2001). This may reflect the fact that iron levels in mitochondria represent only a small fraction of the total iron content in plants and that the *Arabidopsis* genome encodes two additional genes designated *AtSTA*2 and *AtSTA*3 that are similar to *AtSTA*1/*atm*1 and may compensate for the lack of *AtSTA*1.

Evidence suggests that the [Fe-S] clusters of cytoplasmic proteins are pre-assembled in the mitochondrial matrix and exported to the cytosol in yeast (Nakai et al., 1998). As mentioned above, Nfs1p is capable of mediating cytoplasmic [Fe-S] maturation only when present in the mitochondrion. Similar results were seen for IscU (Mühlenhoff and Lill, 2000). These data suggest that the [Fe-S] clusters of cytoplasmic proteins are pre-assembled in the mitochondrial matrix and exported to the cytosol. Genetic studies have shown a strong link between cluster assembly systems and the specific components of the [Fe-S] cluster assembly mechanism in eukaryotes (Pilon et al., 1995; Tong and Rouault, 2000; Léon et al., 2003; Tong et al., 2003a). There is published molecular evidence that distinct [Fe-S] cluster assembly complexes exist in the cytoplasm and mitochondria of humans (Tong and Rouault, 2000), which is further supported by recent findings that alternative splicing of the common mRNA leads to synthesis of two Nfu isoforms that are targeted specifically either to mitochondria, or to the cytoplasm and the nucleus in the mammalian cell (Tong et al., 2003b). Further evidence for subcellular compartmentalization of the eukaryotic nif/isc/suf machinery is provided by the presence of AtNFS1, AtNFS2, and distinct NFU proteins in chloroplasts and mitochondria of *Arabidopsis* (Kushnir et al., 2001; Léon et al., 2002; Pilon-Smits et

al., 2002; Léon et al., 2003) as described below. However, little is known about how clusters are inserted into apoproteins.

The contribution of [Fe-S] proteins to plant metabolism

Proteins have exploited the structural and electronic versatility of [Fe-S] clusters to facilitate a vast array of crucial biological processes. There are multiple examples of [Fe-S] proteins in plants that are involved in important activities such as primary metabolism, hormone biosynthesis, energy production, and the stress response (Jordanov et al., 1992; Imsande, 1999). For example, dihydroxy acid dehydratase, the third enzyme of branched amino acid biosynthesis; glutamine phophoribosyl-pyrophosphate amidotransferase, the first enzyme of the *de novo* purine biosynthetic pathway (Flint and Emptage, 1988; Kim et al., 1995); and aldehyde oxidase, involved in the last biosynthetic step for major plant hormones indole-acetic acid (IAA) and abscisic acid (ABA) (Sekimoto et al., 1997; Seo et al., 2000), are all [Fe-S] proteins. In addition, various [Fe-S] proteins are components of the chloroplast inner envelope protein apparatus, and are directly involved in electron transfer (Imsande, 1999). [Fe-S] proteins also mediate the activation of three enzymes of the Calvin cycle and inactivation of the first enzyme of the pentose phosphate pathway in response to light (Staples et al., 1996; Imsande, 1999).

[Fe-S] proteins are inactivated in PSI and PSII during photoinactivation (Imsande, 1999; Suh et al., 2000). This phenomenon, which results in the loss of net photosynthetic capacity, is caused by light in PSII and factors such as chilling and drought (and exacerbated by light) in PSI. The chlorophyll deficient (yellow) phenotype

of plants stressed by Fe, N, or S deficiency has been proposed to be due to defective [Fe-S] clusters in nitrate reductase, nitrite reductase, and glutamate synthase that lead to inadequate or reduced N assimilation (Imsande, 1999), and in adenosine 5' – phosphosulfate reductase, which catalyzes the key step of sulfate assimilation in higher plants (Kopriva et al., 2001). In addition, the first step of biosynthesis of the osmoprotectant, glycine betaine, is catalyzed by the [Fe-S] protein, choline monooxygenase (Rathinasabapathi et al., 1997). Functional [Fe-S] proteins are thus critical to a wide variety of biochemical and metabolic pathways in plants.

Assembly of [Fe-S] clusters in plants

Although rapid progress has been made toward elucidating [Fe-S] cluster assembly in a variety of organisms over the past ten years, very little is known about this process in plants. Plants present a further challenge because, in addition to mitochondria, they also contain chloroplasts, which have been implicated as another site of [Fe-S] cluster assembly, as discussed below. This suggests that plants may have two different machineries, each of which targets a distinct array of cytosolic proteins. To date, seven nuclear genes have been directly linked to [Fe-S] cluster assembly in plants and have been characterized to some extent. These genes encode the ABC transporter AtSTA1, AtNFS2, and AtNFU1-5 (Kushnir et al., 2001; Léon et al., 2002; Pilon-Smits et al., 2002; Léon et al., 2003), as described below. The focus of this study is on the *AtNFS1, AtISU1, AtISU2, and AtISU3* genes, which are hypothesized to be key players in [Fe-S] cluster biogenesis.

There is good evidence that, as in yeast and animals, plant mitochondria contain a conserved [Fe-S] assembly system in mitochondria that is used to synthesize, assemble, and export [Fe-S] clusters. The N-terminal 54 amino acids of an IscS homolog in *Arabidopsis,* AtNFS1, targets GFP exclusively to mitochondria (Kushnir et al., 2001). Furthermore, a mitochondrial ABC transporter, AtSTA1, that appears to play a role in the mobilization of [Fe-S] clusters to the cytoplasm in plants, complemented a mutation in the yeast transporter, Atm1p, and also targeted GFP to mitochondria (Csere et al., 1998; Bekri et al., 2000a; Kushnir et al., 2001). Recently, five NFU-like proteins designated AtNFU1-5 which show homology to the C-terminus of *NifU* were found to complement a *Δisu1Δnfu1* yeast mutant, which confirmed their role in [Fe-S] cluster biogenesis (Léon et al., 2003). These proteins share a conserved CXXC motif in their NFU domain and can be separated into two classes. AtNFU4 and AtNFU5 have only one NFU-like domain, whose location in the polypeptide is different from that found in AtNFU1-3, and present a structural organization that is similar to *S. cerevisiae* Nfu1p. AtNFU4 localized to mitochondria when fused with GFP (Léon et al., 2003). Further, work done in this project has shown that full-length AtNFS1, AtISU1, and AtISU3 also localize exclusively in mitochondria. It had been hypothesized that the mercaptopyruvate sulfurtransferase (MST) homologs in *Arabidopsis* function in providing the sulfur for cluster assembly by transferring sulfur ions from mercaptopyruvate to cyanide ions (Nakamura et al., 2000; Papenbrock and Schmidt, 2000). One of the two MST's present in *Arabidopsis* is located in mitochondria, however a T-DNA knockout mutant of the gene encoding this enzyme, had no apparent

effect on [Fe-S] enzyme activities, therefore suggesting that this protein is not directly involved in iron-sulfur cluster assembly.

Evidence for [Fe-S] cluster biogenesis in chloroplasts has come from observations that in the presence of ATP and NADPH or light, lysed spinach chloroplasts are able to support the formation of endogenous [Fe-S] clusters targeted for ferredoxin maturation *in vitro,* indicating the existence of [Fe-S] cluster biogenesis machinery in plastids (Takahashi et al., 1991). This finding was confirmed and extended by Pilon et al., (1995). A large portion of the iron in a plant leaf is found in the chloroplasts, where it is assembled into redox-active heme or [Fe-S] clusters of the photosynthetic electron transfer chain (Raven et al., 1999). *AtNFS2* (or *AtCpNIFS*) is one of two sequences in *Arabidopsis* with homology to the *nifS/iscS/sufS* family. Phylogenetic analysis of this gene shows a distant relationship to IscS and NifS and places it in a heterologous group of proteins more homologous to SufS (Tachezy et al., 2001). The chloroplast location of AtNFS2 is supported by ChlorP, PSORT, and TargetP analysis and experiments tracking localization of the N-terminal one-third of the protein fused to GFP in *Arabidopsis* protoplasts as well as *in vitro* chloroplast import assays of the full-length protein (Léon et al., 2002; Pilon-Smits et al., 2002). Similar to *E. coli* SufS, both selenocysteine and cysteine can be used as substrates by AtNFS2 and higher activity is seen towards the Se substrate *in vitro* (Pilon-Smits et al., 2002). The similarity of AtNFS2 in sequence and properties to SufS suggests that the physiological function of AtNFS2 is distinct from or in addition to [Fe-S] cluster assembly and that AtNFS2 could participate in the SUF pathway for [Fe-S] cluster assembly in the chloroplast. It is interesting to note that, in addition to AtNFS2, *Arabidopsis* appears to

contain all the other components of the SUF pathway (Table I). All of the *Arabidopsis* Suf-like proteins contain N-terminal extensions that suggest targeting to chloroplasts by the program ChlorP. These observations indicate that the SUF-like pathway for [Fe-S] cluster assembly is operative in the chloroplast. In addition, the AtNFU1-3 proteins also localize to chloroplasts as confirmed by GFP localization to the N-terminus of the proteins (Léon et al., 2003). These proteins represent a new class of NFU proteins that have two NFU domains and are more related to *Synechocystis* PCC6803 NFU-like proteins which are capable of accepting and transferring a [2Fe-2S] cluster to an apoferredoxin (Mühlenhoff et al., 2002). Work done in this project suggests that AtISU2 is also localized to chloroplasts.

The uncharacterized genes of plant [Fe-S] cluster biogenesis

Initial analysis of the *Arabidopsis* genome has indicated that at least 40 genes may be involved in some aspect of the [Fe-S] cluster biogenesis (B. Winkel, D. Dean, and T. Larson, unpublished findings) and many more proteins are dependant for their function on the activity of these clusters. Table I lists *Arabidopsis* genes with predicted protein products homologous to the [Fe-S] cluster biogenesis machinery in other organisms. As described above, only 7 of these have been studied to any significant extent.

Among the as-yet-uncharacterized genes, AtNFS1, which bears homology to the nifS/iscS/sufS family is of particular interest. The predicted protein is most closely related to NifS, IscS, and mitochondrial homologs that have known cysteine desulfurase activity (Tachezy et al., 2001). As mentioned earlier, the N-terminal sequences of

Table I. Members of the *Nif/Isc/Suf* families that have been identified in bacteria, yeast, and *Arabidopsis*, with genomic information for the *Arabidopsis* homologs.

(B. Winkel, D. Dean, and T. Larson, unpublished data).

Function(s)	**Bacterial protein(s)**	**Yeast Homo-log(s)**	***Arabidopsis* Homolog(s)[1]**	**Cellular location[2]**	***Arabidopsis* EST matches**	**Salk T-DNA lines**
cysteine desulfurase (seleno-cysteine lyase)	NifS, IscS, SufS	Nfs1p	AtNFS1 (At5g65720)	M	12 (green siliques, flowers, pooled mRNA[3])	3
			AtNFS2 (At1g08490)	C	8 (green siliques, roots, dehydration- and cold-treated rosettes and plants at various stages)	4
			ABA3 (At1g16540)	Cyt.		1
scaffold for cluster assembly.	NifU (N-terminal domain), IscU	Isu1p, Isu2p	AtISU1 (At4g22220)	C	8 (seedlings, leaves, roots, flowers, dehydration- and cold-treated plants at various stages)	1
			AtISU2 (At4g04080)	C		1
			AtISU3 (At3g01020)	MC	0 in database, 1 cDNA from flowers (gift of Stephane Lobreaux)	0
					0 in database, 1 cDNA from flowers (gift of Stephane Lobreaux)	
Unknown, possibly dispensable in yeast	NifU (C-terminal domain)	Nfu1p	AtNFU1 (At4g01940)	c	11 (dehydration- and cold-treated rosettes and plants at various stages, pooled mRNA[3])	1
			AtNFU2 (At4g25910)	c	1 (tissue source unknown)	0
			AtNFU3 (At5g49940)	c	4 (tissue source unknown)	2
			AtNFU4 (At1g51390)	c	2 (dehydration- and cold-treated plants at various stages, pooled mRNA[3])	0
			AtNFU5 (At3g20970)	c	6 (developing seed, green siliques, rosette plants, pooled mRNA[3])	2
alternate scaffold for cluster assembly	IscA, HesB, SufA	Isa1p, Isa2p	AtISA1 (At2g16710)*	-	2 (root)	0
			AtISA2 (At2g36260)*	m	0	0
			AtISA3 (At1g10500)*	c	9 (2-6 wk above-ground, 2-3 wk leaves, 12 wk leaves, dehydration- and cold-treated rosettes and plants at various stages, pooled mRNA[3])	1
			AtISA4 (At5g03905)*	-	0	0

feedback regulation of cluster bio-synthesis	IscR	None	None	N/A[4]	N/A	N/A
reduction of sulfur, iron, or Fe-S cluster inter-mediate	Fdx ([2Fe-2S]-ferredoxin)	Yah1p	AtYAH1 (At4g05450) AtYAH2 (At4g21090)		1 (green siliques) 5 (roots, flower buds)	1 0
Hsp70/ DnaK chaperon	HscA	Ssq1p	At5g09590 At5g49910 At1g16030, many others	M C	11 13 3	
Hsp40/ DnaJ Chaperone	HscB	Jac1p	At5g06410	c	N/A	N/A
NAD(P)H-dependent reduction of ferredoxin	Ferredoxin reductase	Arh1p	AtARH1 (At4g32360)		3 (green siliques, plants at various stages)	1
ABC transporter	SufC, Atm1 (*Rickettsia prowazekii*)	Atm1p	STA1 (At5g58260)	M	6 (flower buds, inflorescence, cold-treated rosettes, plants at various stages, pooled mRNA[3])	1
			STA2 (At4g28620)		0	1
			STA3 (At4g28630)		4 (roots, plants at various stages)	1
	SufC SufB/D		AtSFC	c		
	SufE		AtSFB1 (At4g04770)	c	6 (roots, plants at various stages)	3
			AtSFB2 (At1g32500)	c	3 (rosette plants, plants at various stages)	1
			AtSFE1 (At4g26500)	c		1
			AtSFE2 (At5g50210)	c	2 (silique and flower)	0
			AtSFE3 (At1g67810)	c	5 (roots, seeds, green siliques, plants)	0

Rhodanese-like proteins						
Mercapto-pyruvate sulfur-transferase	SseA	None	AtMST1 (At1g79230)	m, c	18 (various)	0
			AtMST2 (At1g16460)	cytopl.	10 (various yes)	1
	YceA		AtYCA1 (At2g40760)	c	0	4
			AtYCA2 (At1g17850)	c	1 (nomixture)	0
Unknown			AtYCA3 (At1g09280)	-	4 (various, mixture)	0
	PpiC, NifM		AtPPIR (At5g19370)		2 (rosettes, roots; PPIR = PPI-rhodanese fusion)	0
Peptidyl-prolyl *cis/trans* isomerase, rhodanese	GlpE, PspE		AtSEN1 (At4g35770)	c	57 (various, mixture)	2
			AtGlpE1 (At5g66040)	c	15 (various, mixture)	0
			AtGlpE2 (At2g17850)	-	0	1
			AtGlpE3 (At2g21045)	-	0	2
			AtGlpE4 (At5g66170)	-	6 (various, mixture)	0
			AtGlpE5 (At4g27700)	-	4 (various, mixture, rosette plants)	1
	MoeB		AtGlpE6 (At2g42220)	c	8 (mixture)	1
			AtMOC3 (At5g55130)	c	6 (various, mixture)	0

[1]numbers in parentheses are accession numbers for the corresponding protein sequences.
[2]C or M indicates a chloroplast or mitochondrial location, respectively (experimental determination).
Lower case "c", "m" or – indicates a chloroplast, mitochondrial, or unpredicted location, respectively, predicted by ChlorP and/or TargetP.
[3]from 7 day germinated etiolated seedlings; tissue culture grown roots; rosettes, stems, flowers and siliques from staged plants half grown in 24 h light cycle, half in16 h light, 8 h dark.
[4]N/A = not applicable.

AtNFS1 has been shown to target GFP to mitochondria, but no other information has been published on this protein. Besides AtNFU1-5, there are several other proteins that are likely candidates of scaffold proteins, namely three genes homologous to *IscU* (66-67% amino acid identity) and four genes with homology to *IscA* (30-40%). The *AtISU* and *AtISA* genes have the highest sequence identity with known [Fe-S] cluster assembly proteins. Multiple proteins with rhodanese domains are also found in *Arabidopsis* (Table I), which are all predicted to be targeted to the chloroplast. It is known that IscS transfers sulfur to the rhodanese domain of the enzyme, ThiI, during synthesis of 4-thiouridine of tRNAs and the thiazole ring of thiamin in *E. coli* (Palenchar et al., 2000; Mueller et al., 2001; Wright et al., 2002). The high degree of apparent redundancy in rhodanese domains may be significant in that these proteins may interact with and accept sulfur from AtNFS2 and subsequently transfer it to various targets.

Arabidopsis also contains genes that bear homology to the ferredoxins and heat shock proteins of the *nifS/iscS/sufS* operons. In addition, the ABA3 locus in *Arabidopsis* defines a protein in which a sulfurtransferase domain is fused to another functional domain which catalyzes the activation of the molybdenum enzymes, aldehyde oxidase and xanthine dehydrogenase (Bittner et al., 2001). Whether this protein functions in [Fe-S] cluster assembly is being investigated (Florian Bittner, personal communication).

Hypothesis

Work described in this dissertation tested the hypothesis that the *Arabidopsis* *AtNFS1*, *AtISU1*, *AtISU2* and *AtISU3* genes play an essential role in [Fe-S] cluster

biogenesis in either mitochondria or chloroplasts and that these genes are differentially regulated at the transcriptional level in a developmental or tissue-specific manner.

Project objectives

There are approximately 40 genes in *Arabidopsis* that could be involved in some aspect of [Fe-S] cluster biogenesis, and there are numerous other proteins whose function depends on the activity these genes. The objective of the proposed project is to begin characterizing the *AtNFS1, AtISU1*, *AtISU2,* and *AtISU3* genes and to begin defining physiological processes for which they are important. Proteins encoded by these genes show high homology to NifS/IscS and NifU/IscU respectively, and are most likely, key players in the plant [Fe-S] cluster assembly mechanism. To test the hypothesis the following specific objectives were proposed: (1) determine the subcellular localization of the AtNFS1, AtISU1, AtISU2 and ISU3 proteins, (2) begin determining the physiological functions influenced by AtNFS1, AtISU1, AtISU2, and AtISU3 proteins using overexpression and down regulation strategies and, (3) characterize the tissue-specific expression of the *AtNFS1, AtISU1, AtISU2,* and *AtISU3* genes.

Rationale and significance of proposed study

The significance of the [Fe-S] cluster biogenesis process is reflected in the observation that failure of the isc machinery appears to be even more detrimental to the survival of the eukaryotic cell than defects in other well-known processes in mitochondria. For example, genes encoding components of oxidative phosphorylation

could be deleted in yeast and cells could survive, provided that substrates supporting growth by fermentation were available (Tzalgoloff and Dieckmann, 1990; Grivell et al., 1999). However, cells lacking the ability to synthesize [Fe-S] clusters cannot be rescued because it is not possible to provide these clusters as substrates.

Extraordinary sequence conservation among the genes of the isc machinery in all organisms studied to date suggests that the mechanism of [Fe-S] cluster assembly and insertion into apoproteins is highly conserved and ubiquitous, although many questions remain, particularly in plants. The major goal of this project is to determine how plants assemble and distribute [Fe-S] clusters to the many different [Fe-S] proteins that are essential for different reactions in different cellular compartments – the cytoplasm, mitochondria, chloroplasts, and the nucleus. In *A. vinelandii* there is one set of [Fe-S] biosynthetic proteins specific for maturation of the abundant nitrogenase catalytic components and another set of homologous proteins involved in maturation of other cellular [Fe-S] proteins. In *E. coli*, there are two distinct [Fe-S] cluster assembly mechanisms, the isc machinery which is the "housekeeping" machinery and the suf machinery which plays a role during iron depletion and oxidative stress. By analogy, it is possible that the function of different ISU proteins in *Arabidopsis* is to provide the specific delivery of [Fe-S] cluster precursors to different locations. This makes physiological sense because this can provide the plant with the ability to control the activation of [Fe-S] proteins in a hierarchic fashion. For example, one way that a plant responds to specific stress is to preserve certain physiological functions at the expense of others. Because [Fe-S] proteins are involved in so many fundamental aspects of plant physiology, the controlled distribution of [Fe-S] clusters could be one mechanism

of regulation. The long-term goal of this project is to develop a model for the mechanism of [Fe-S] cluster formation in plants and to determine whether this system has a role in regulating plant growth and responses to the environment. This information could be helpful from the perspective of metabolic engineering to enhance plant productivity by modifying the distribution and balance of active [Fe-S] proteins.

REFERENCES

Agar, J.N., Zheng, L., Cash, V.L., Dean, D.R., and Johnson, M.K. (2000a). Role of the IscU protein in iron-sulfur cluster biosynthesis: IscS-mediated assembly of a [Fe2S2] cluster in IscU. JACS Comm.**,** 2136-2137.

Agar, J.N., Krebs, C., Frazzon, J., Huyhn, 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] cluster in IscU. Biochemistry **39,** 7856-7862.

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

Allikmets, R., Raskind, W.H., Hutchinson, A., Schueck, N.D., Dean, M., and Koeller, D.M. (1999). Mutation of a putative mitochondrial iron transporter gene (ABC7) in X-linked sideroblastic anemia and ataxia (XLSA/A). Hum. Mol. Gen. **8,** 743-749.

Amrani, L., Primus, J., Glatigny, A., Arcangeli, L., Scazzocchio, C., and Finnerty, V. (2000). Comparison of the sequences of the *Aspergillus nidulans hxB* and *Drosophila melanogaster ma-l* genes with *nifS* from *Azotobacter vinelandii* suggests a mechanism for the insertion of the terminal sulphur atom in the molybdopterin cofactor. Mol. Microbiol. **38,** 114-125.

Andersson, S.G., Zomorodipour, A., Andersson, J. O., Silcheritz-Ponten, Alsmark, U. C., Podowski, R. M., Nasalund, A. K., Eriksson, E. S., Winkler, H. H., and

Kurland, C. G. (1998). The genome sequence of Rickettsia prowazekii and the origin of mitochondria. Nature **396,** 133-140.

Bekri, S., Kispal, G., Lange, H., Fitzimonis, E., Tolmie, J., Lill, R., and Bishop, D.F. (2000a). Human ABC7 transporter: gene structure and mutation causing X-linked sideroblastic anemia with ataxia with disruption of cytosolic iron-sulfur protein maturation. Blood **9,** 3256-3264.

Bekri, S., Kispal, G., Lange, H., Fitzsimons, E., Tolmie, J., Lill, R., and Bishop, D.F. (2000b). Human ABC7 transporter: gene structure and mutation causing X-linked sideroblastic anemia with ataxia with disruption of cytosolic iron-sulfur protein maturation. Blood **96,** 3256-3264.

Bittner, F., Oreb, M., and Mendel, R.R. (2001). ABA3 is a molybdenum cofactor sulfurase required for activation of aldehyde oxidase and xanthine dehydrogenase in *Arabidopsis thaliana.* J. Biol. Chem. **276,** 40381-40384.

Clausen, T., Kaiser, J. T., Steegborn, C., Huber, R., and Kessler, D. Proc Natl Acad Sci USA. (2000). Crystal structure of the cystine C-S lyase from Synechocystis: stabilization of cysteine persulfide for FeS cluster biosynthesis **8,** 3856-3861.

Craig, E.A., Voisine, C., and Schilke, B. (1999). Mitochondrial iron metabolism in the yeast Saccharomyces cerevisiae, **Biol Chem,** 1167-1173.

Csere, P., Lill, R., and Kispal, G. (1998). Identification of a human mitochondrial ABC transporter, the functional orthologue of yeast Atm1p. FEBS Lett **441,** 266-270.

Ellis, K.E., Clough, B., Saldanha, J.W., and Wilson, R.J. (2001). NifS and SufS in malaria. Mol. Microbiol. **41,** 973-981.

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

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

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

Fujii, T., Maeda, M., Mihara, H., Kurihara, T., Esaki, N., and Hata, Y. (2000). Structure of a NifS homologue: X-ray structure analysis of CsdB, an Escherichia coli counterpart of mammalian selenocysteine lyase. Biochemistry **39,** 1263-1273.

Garland, S.A., Hoff, K., Vickery, L. E., and Culotta, V. C. (1999). Saccharomyces cerevisiae ISU1 and ISU2: members of a well-conserved gene family for iron-sulfur cluster assembly. J Mol Bio **294,** 897-907.

Grivell, L.A., Artal-Sanz, M., Hakkaart, G., de Long, L., Nijtmans, L.G., van Oosterum, K., Siep, M., and van der Spek, H. (1999). Mitochondrial assembly in yeast. FEBS Lett **452,** 57-60.

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. U.S.A. **97,** 7790-7795.

Hwang, D.M., Dempsey, A., Tan, K. T., and Liew, C. C. (1996). A modular domain of NifU, a nitrogen fixation cluster protein, is highly conserved in evolution. J Mol Evol **43,** 536-540.

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

Jacobson, M.R., Cash, V.L., Weiss, M.C., Laird, N.F., Newton, W.E., and Dean, D.R. (1989). Biochemical and genetic analysis of the *nifUSVWZM* cluster from *Azotobacter vinelandii*. Mol . Gen. Genet. **219,** 49-57.

Jacobson, M.R., Brigle, K. E., Bennett, L. T., Setterquist, R. A., Wilson, M. S., Cash, V. L., J., B., Newton, W. E., and Dean, D. R. (1989a). FILL-IN !! J Bacteriol **171,** 1017-1027.

Jaschkowitz, K., and Seidler, A. (2000). Role of a NifS-like protein from the cyanobacterium *Synechocystis* PCC 6803 in the maturation of FeS proteins. Biochem. **39,** 3416-3423.

Jordanov, J., Courtois-Verniquet, F., Neuburger, M., and Douce, R. (1992). Structural investigations by extended X-ray absorption fine structure spectroscopy of the iron center of mitochondrial aconitase in higher plant cells. J. Biol. Chem. **267,** 16775-16778.

Kaiser, J.T., Clausen, T., Bourenkow, G. P., Bartunik, H. D., Steinbacher, S., and Huber, R. , . (2000). Crystal structure of a NifS-like protein from Thermotoga maritima: implications for iron sulphur cluster assembly. J Mol Bio **297,** 133-140.

Kambampati, R., and Lauhon, C.T. (2000). Evidence for the transfer of sulfane sulfur from IscS to ThiI during the *in vitro* biosynthesis of 4-thiouridine in *Escherichia coli* tRNA. J. Biol. Chem. **275,** 10727-10730.

Kato, S., Mihara, H., Kurihara, T., Yoshimura, T., and 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., and 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.

Kim, J.H., Delauney, A.J., and Verma, D.P. (1995). Control of de novo purine biosynthesis genes in ureide-producing legumes: induction of glutamine phosphoribosylpyrophosphate aminodtransferase gene and characterization of its cDNA from soybean and Vigna. Plant J. **7,** 77-86.

Kispal, G., Csere, P., Prohl, C., and Lill, R. (1999). The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. EMBO J. **18,** 3981-3939.

Kopriva, S., Büchert, T., Fritz, G., Suter, M., Weber, M., Benda, R., Schaller, J., Feller, U., Schürmann, P., Schünemann, V., Trautwein, A.X., Kroneck, P.M.H., 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.

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.

Lange, H., Lisowsky, T., Gerber, J., Muhlenhoff, U., Kispal, G., and Lill, R. (2001). An essential function of the mitochondrial sulfhydryl oxidase Erv1p/ALR in the maturation of cytosolic Fe/S proteins. EMBO Rep. **2,** 715-720.

Lauhon, C.T. (2002). Requirement for IscS in biosynthesis of all thionucleosides in *Escherichia coli*. J. Bacteriol. **184,** 6820-6829.

Lauhon, C.T., and Kambampati, R. (2000). The *iscS* gene in *Escherichia coli* is required for the biosynthesis of 4-thiouridine, thiamin, and NAD. J. BIol. Chem. **275,** 20096-20103.

Leibrecht, I., and Kessler, D. (1997). A novel L-cysteine/cystine C-S-lyase directing [2Fe-2S] cluster formation of *Synechocystis* ferredoxin. J. Biol. Chem. **272,** 10442-10447.

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

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

Li, J., Kogan, M., Knight, S.A., Pain, D., and Dancis, A. (1999). Yeast mitochondrial protein, Nfs1p, coordinately regulates iron-sulfur cluster proteins, cellular iron uptake, and iron distribution. J Biol Chem **274,** 33025-33034.

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

Lill, R., Diekert, K., Kaut, A., Lange, H., Pelzer, W., Prohl, C., and Kispal, G. (1999). The essential role of mitochondria in the biogenesis of cellular iron-sulfur proteins. Biol Chem. **380,** 1157-1166.

Lima, C.D. (2002). Analysis of the E. coli NifS CsdB protein at 2.0 A reveals the structural basis for perselenide and persulfide intermediate formation. J. Mol. Biol. **315,** 1199-1208.

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.

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.

Marquet, A., Bui, B.T., and Florentin, D. (2001). Biosynthesis of biotin and lipoic acid. Vitam. Horm. **61,** 51-101.

Masepohl, B., Angermuller, S., Hennecke, S., Hubner, P., Moreno-Vivian, C., and Klipp, W. (1993). Nucleotide sequence and genetic analysis of the *Rhodobacter capsulatus* ORF6-*nifUI* SVW gene region: possible role of *NifW* in homocitrate processing. Mol. Gen. Genet. **238,** 369-382.

Merchant, S., and Dreyfuss, B.W. (1998). Posttranslational assembly of photosyntheic metalloproteins. Annu Rev Plant Physiol Plant Mol Biol **49,** 25-51.

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

Mihara, H., Maeda, M., Fujii, T., Kurihara, T., Hata, Y., and Esaki, N. (1999). A nifS-like gene, csdB, encodes an Escherichia coli counterpart of mammalian selenocysteine lyase. Gene cloning, purification, characterization and preliminary x-ray crystallographic studies. J. Biol. Chem. **274,** 14768-14772.

Mueller, E.G., Palenchar, P.M., and Buck, C.J. (2001). The role of the cysteine residues of ThiI in the generation of 4-thiouridine in tRNA. J. Biol. Chem. **276,** 33588-33595.

Mühlenhoff, U., and Lill, R. (2000). Biogenesis of iron-sulfur proteins in eukaryotes: a novel task of mitochondria that is inherited from bacteria. Biochim. Biophys. Acta. **1459,** 370-382.

Mühlenhoff, U., Richhardt, N., Gerber, J., and Lill, R. (2002). Characterization of iron-sulfur protein assembly in isolated mitochondria. J. Biol. Chem. **277,** 29810–29816.

Muller, K., Matzanke, B.F., Schunemann, V., Trautwein, A.X., and Hantke, K. (1998). FhuF, an iron-regulated protein of *Escherichia coli* with a new type of [2Fe-2S] center. Eur. J. Biochem. **258,** 1001-1008.

Nakai, Y., Yoshihara, Y., H., H., and Kagamiyama, H. (1998). cDNA cloning and characterization of mouse nifS-like protein, m-Nfs1: mitochondrial localization of eukaryotic NifS-like proteins. FEBS Lett **433,** 143-148.

Nakamura, M., Saeki, K., and Takahashi, J. (1999). Hyperproduction of recombinant ferredoxins in Escherichia coli by coexpression of the ORF1-ORF2-iscS-iscU-iscA-hscB-hs cA-fdx-ORF3 gene cluster. J Biochem (Tokyo) . **126,** 10-18.

Nakamura, T., Yamaguchi, Y., and Sano, H. (2000). Plant mercaptopyruvate sulfurtransferases. Molecular cloning, subcellular localization and enzymatic activities. Eur. J. Biochem. **267,** 5621-5630.

Nakamura, Y., Kaneko, T., Sato, S., Ikeuchi, M., Katoh, H., Sasamoto, S., Watanabe, A., Iriguchi, M., Kawashima, K., Kimura, T., Kishida, Y., Kiyokawa, C., Kohara, M., Matsumoto, M., Matsuno, A., Nakazaki, N., Shimpo, S., Sugimoto, M., Takeuchi, C., Yamada, M., and Tabata, S. (2002). Complete genome structure of the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1. DNA Res. **9,** 123-130.

Nilsson, K., Lundgren, H.K., Hagervall, T.G., and Bjork, G.R. (2002). The cysteine desulfurase IscS is required for synthesis of all five thiolated nucleosides present in tRNA from *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **184,** 6830-6835.

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

Ollagnier-de-Choudens, S., Mattioli, T., Takahashi, Y., and 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, 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 E. coli. J. Biol. Chem. **in press**.

Palenchar, P.M., Buck, C.J., Cheng, H., Larson, T.J., and Mueller, E.G. (2000). Evidence that ThiI, an enzyme shared between thiamin and 4-thiouridine biosynthesis, may be a sulfurtransferase that proceeds through a persulfide intermediate. J. Biol. Chem. **275,** 8283-8286.

Papenbrock, J., and Schmidt, A. (2000). Characterization of a sulfurtransferase from *Arabidopsis thaliana*. Eur. J. Biochem. **267,** 145-154.

Patzer, S.I., and Hantke, K. (1999). SufS is a NifS-like protein, and SufD is necessary for stability of the [2Fe-2S] FhuF protein in *Escherichia coli*. J. Bacteriol. **181,** 3307-3309.

Pelzer, W., Mühlenhoff, U., Diekert, K., Siegmend, K., Kispal, G., and Lill, R. (2000). Mitochondrial Isa2p plays a crucial role in the maturation of iron-sulfur proteins. FEBS Lett. **476,** 134-139.

Pilon, M., America, T., van't Hof, R., de Kruiff, B., and Weisbeek, P. (1995). Protein translocation into chloroplasts. In Advances in Molecular and cell Biology: 1. Membrane Protein Transport, S.S. Rothman, ed (Greenwich, CT: JAI Press), pp. 229-255.

Pilon-Smits, E.A., 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.

Rathinasabapathi, B., Burnet, M., Russell, B.L., Gage, D.G., 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.

Raven, J.A., Evans, M.C.A., and Korb, R.E. (1999). The role of trace metals in photosynthetic electron transport in O2-evolving organisms. Photosynth. Res. **60,** 111-149.

Schilke, B., Voisine, C., Beinert, H., and Craig, E. (1999). Evidence for a conserved system for iron metabolism in the mitochondria of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **96,** 10206-10211.

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.

Sekimoto, H., Seo, M., Dohmaei, N., Takioi, K., Kamiya, Y., and Koshiba, T. (1997). Cloning and Molecular Characterization of Plant Aldehyde Oxidase. J. Biol. Chem. **272,** 15280–15285.

Seo, M., Peeters, A.J., Koiwai, H., Oritani, T., Marion-Poll, A., Zeevaart, J.A., Koornneef , M., Kamiya, Y., and Koshiba, T. (2000). The Arabidopsis aldehyde

oxidase 3 (AAO3) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. Proc. Natl. Acad. Sci. USA **97,** 12908-12913.

Staples, C.R., Ameyibor, E., Fu, W., Gardet-Salvi, L., Stritt-Etter, A.-L., Schürmann, P., Knaff, D.B., and Johnson, M.K. (1996). The function and properties of the iron-sulfur center in spinach ferredoxin:thioredoxin reductase: A new biological role for iron-sulfur clusters. Biochem. **35,** 11425-11434.

Suh, H.J., Kim, C.S., and Jung, J. (2000). Cytochrome b6/f complex as an indigenous photodynamic generator of singlet oxygen in thylakoid membranes. Photochem. Photobiol. **71,** 103-109.

Sun, D., and Setlow, P. (1993). Cloning, nucleotide sequence, and regulation of the *Bacillus subtilis nadB* gene and a *nifS*-like gene, both of which are essential for NAD biosynthesis. J. Bacteriol. **175,** 1423-1432.

Tachezy, J., Sanchez, L.B., and Muller, M. (2001). Mitochondrial type iron-sulfur cluster assembly in the amitochondriate eukaryotes *Trichomonas vaginalis* and *Giardia intestinalis*, as indicated by the phylogeny of IscS. Mol. Biol. Evol.**,** 1919-1928.

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 homolgs in Archaea and plastids. J. Biol. Chem. **277,** 28380-28383.

Takahashi, Y., Mitsui, A., and Matsubara, H. (1991). Formation of the Fe-S cluster of ferredoxin in lysed spinach chloroplasts. Plant Physiol. **95,** 97-103.

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

Tokumoto, U., Nomura, S., Minami, Y., Mihara, H., Kato, S., Kurihara, T., Esaki, N., Kanazawa, H., Matsubara, H., and Takahashi, Y. (2002). Network of protein-protein interactions among iron-sulfur cluster assembly proteins in *Escherichia coli*. J. Biochem. **131,** 713-719.

Tong, W.H., and Rouault, T. (2000). Distinct iron-sulfur cluster assembly complexes exist in the cytosol and mitochondria of human cells. EMBO J. **19,** 5692-5700.

Tong, W.H., Jameson, G.N., Huynh, B.H., and Rouault, T.A. (2003a). Subcellular compartmentalization of human Nfu, an iron-sulfur cluster scaffold protein, and its ability to assemble a [4Fe-4S] cluster. Proc Natl Acad Sci U S A **100,** 9762-9767.

Tong, W.H., L., J.G.N., Huyhn, B.H., and Rouault, T.A. (2003b). Subcellular compartmentalization of human Nfu, an iron-sulfur cluster scaffold protein, and its ability to assemble a [4Fe-4S] cluster. Proc Natl Acad Sci U S A.

Tzalgoloff, A., and Dieckmann, C.L. (1990). PET genes of Saccharomyces cerevisiae. Microbiol Rev **54,** 211-225.

Wollenberg, M., Berndt, C., Bill, E., Schwenn, J.D., and 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.

Wright, C.M., Palenchar, P.M., and Mueller, E.G. (2002). A paradigm for biological sulfur transfers via persulfide groups: a persulfide-disulfide-thiol cycle in 4-thiouridine biosynthesis. Chem. Comm.**,** 2708-2709.

Xi, J., Ge, Y., Kinsland, C., McLafferty, F.W., and Begley, T.P. (2001). Biosynthesis of the thiazole moiety of thiamin in *Escherichia coli*: identification of an acyldisulfide-linked protein--protein conjugate that is functionally analogous to the ubiquitin/E1 complex. Proc Natl Acad Sci U S A **98,** 8513-8518.

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., White, R.H., Cash, V.L., and Dean, D.R. (1994). Mechanism for the desulfurization of L–cysteine catalyzed by the *nifS* gene product. Biochemistry **33,** 4714–4720.

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.

CHAPTER 2

AtNFS1 mediates iron-sulfur cluster assembly in *Arabidopsis* mitochondria

Ana Paula Guedes Frazzon[1], Ujwala Warek[2], Jeverson Frazzon[3], Dennis R. Dean[4] and Brenda S. J. Winkel[2]

Graduate Program in Cellular and Molecular Biology (PPGBCM/Cbiot)[1], Institute of Food Science[3], Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil and Departments of Biology[2] and Biochemistry[4], Virginia Tech, Blacksburg, VA. USA

ABSTRACT

The *Arabidopsis AtNFS1* gene encodes a protein with high homology to the group I NifS/IscS/SufS-like proteins that function in [Fe-S] cluster biogenesis and other aspects of sulfur trafficking in cells. This gene has the potential to impact processes as diverse as the energy-producing systems of respiration and photosynthesis, phytohormone biosynthesis, and environmental sensing, as well as general sulfur homeostasis. AtNFS1 was shown to have cysteine desulfurase activity and to be capable of [Fe-S] cluster biogenesis *in vitro* using either *Azotobacter vinelandii* IscU or a putative *Arabidopsis* ISU protein as the scaffold for cluster assembly. AtNFS1 was confirmed to be located exclusively in mitochondria and transgenic plants expressing *AtNFS1* in the antisense orientation exhibited phenotypes consistent with a role for this gene in hormone biosynthesis and the response to oxidative stress. These results provide the first biochemical evidence for a [Fe-S] cluster assembly system in plants and, together with recent findings on the *AtSTA1, AtNFS2,* and *AtNFU1-5* genes, support the existence of distinct systems for sulfur trafficking in mitochondria and chloroplasts.

INTRODUCTION

Iron-sulfur [Fe-S] clusters are extremely versatile protein cofactors that appear to be used by all living organisms to perform a wide variety of biological functions (reviewed in Beinert, 2000; Gerber and Lill, 2002; Frazzon and Dean, 2003; Kiley and Beinert, 2003). More than 100 different [Fe-S] proteins have been identified to date, with roles in three major cellular processes – respiration, photosynthesis, and nitrogen fixation – as well as numerous other metabolic systems including the TCA cycle, in the regulation of gene expression, and in environmental sensing. Plants produce a number of [Fe-S] proteins not found in other organisms, including enzymes required for synthesis of the osmoprotectant, glycine betaine, and the phytohormones, indole-3-acetic acid and abscisic acid (Rathinasabapathi et al., 1997; Sekimoto et al., 1998; Seo et al., 2000; Hibino et al., 2002), as well as components of the chloroplast inner envelope protein import apparatus, the proteins that mediate electron transfer in photosystem I (PSI), the cytochrome b6/f complex of photosystem II (PSII), and the first energy-coupling site of the mitochondrial respiratory chain (reviewed in Imsande, 1999). Novel [Fe-S] proteins continue to be identified in plants, such as a recently-described adenosine 5'-phosphosulfate reductase, which catalyzes a key step in higher plant sulfate assimilation (Kopriva et al., 2001). Thus, many key aspects of plant metabolism, including all of the basic energy metabolism pathways and systems required for growth and development, the assimilation of nutrients, and the response to stress, depend on functional [Fe-S] proteins.

Although a great deal is known about the structural and functional aspects of [Fe-S] proteins, the details of how [Fe-S] clusters are formed and inserted into target

proteins have only recently emerged from studies on bacterial and yeast systems. The first insights came from studies of the nitrogenase enzyme in *Azotobacter vinelandii* (Zheng et al., 1993; Fu et al., 1994). This work identified two key protein partners, designated NifS and NifU, that are encoded within a *nif* operon. NifS is a pyridoxal 5'-phosphate (PLP)-dependent L-cysteine desulfurase that mobilizes sulfur from L-cysteine to provide the inorganic sulfur necessary for [Fe-S] cluster formation. NifU is an Fe-binding protein that serves as a scaffold for intermediate cluster assembly (Yuvaniyama et al., 2000a). Sulfur is delivered from NifS to NifU, upon which an [Fe-S] cluster is formed and then transferred to the nitrogenase component proteins.

A. vinelandii also produces a second set of homologous proteins that are responsible for general, or "housekeeping" [Fe-S] cluster assembly (Zheng et al., 1998). These proteins are designated IscS (homologous to NifS) and IscU (homologous to the N terminus of NifU) to indicate their roles in the general iron-sulfur-cluster (ISC) assembly process. In addition to IscS and IscU, the operon contains genes for two heat-shock cognate proteins and three other proteins, IscA, IscR, and ferredoxin. The hsc proteins are required for full activation of [Fe-S] proteins, although their specific functions remain to be determined (Zheng et al., 1998; Hoff et al., 2000; Tokumoto and Takahashi, 2001). The function of ferredoxin in [Fe-S] cluster biogenesis is also not yet known. IscA appears to function as an alternative scaffold for [Fe-S] cluster assembly (Krebs et al., 2001), while IscR is involved in negative feedback transcriptional regulation of the *isc* operon (Frazzon and Dean, 2001; Schwartz et al., 2001).

A third system for [Fe-S] cluster assembly, designated *suf* for its role in the mobilization of sulfur, was recently discovered in *E. coli* (Takahashi and Tokumoto,

2002; Outten et al., 2003). The *suf* operon is regulated by oxidative stress and iron status, and is required for the stability of certain [Fe-S] proteins (Muller et al., 1998; Patzer and Hantke, 1999). These observations provide strong support for a role of the SUF machinery in [Fe-S] cluster biogenesis or repair, since a major consequence of oxidative stress is damage to [Fe-S] clusters.

It has become apparent that the NIF/ISC/SUF systems are part of a highly-conserved mechanism for [Fe-S] cluster biogenesis that is used by most organisms, including Eubacteria, Archaea, yeast, animals, and plants (Kushnir et al., 2001; Frazzon et al., 2002; Gerber and Lill, 2002; Mihara and Esaki, 2002; Frazzon and Dean, 2003). Homologs to the components of the eubacterial systems have been found in almost all organisms examined to date and significant genetic and biochemical characterization has been performed in some cases, particularly in cyanobacteria, yeast, and humans. Many organisms contain both ISC- and SUF-like systems, while others, like *A. vinelandii*, lack the SUF machinery (Mihara and Esaki, 2002). Other organisms appear to use an assembly mechanism that is either partially or entirely different, suggesting that there are additional mechanisms for the biogenesis of [Fe-S] clusters that remain to be characterized (Leibrecht and Kessler, 1997; Takahashi and Tokumoto, 2002; Wollenberg et al., 2003).

In eukaryotic cells, [Fe-S] proteins are found in the nucleus, the cytoplasm, mitochondria, and chloroplasts. In yeast, [Fe-S] clusters are assembled in mitochondria and then inserted into resident proteins or exported for maturation of [Fe-S] proteins in the cytoplasm (reviewed in Gerber and Lill, 2002). Among the proteins involved in this process are functional counterparts of bacterial NifS/IscS, the NifU C-terminus, NifU N-

terminus/IscU, IscA, ferredoxin, and two heat shock cognate proteins. The inner membrane ABC transporter, Atm1p, and a sulfhydryl oxidase of the mitochondrial intermembrane space are required for export of [Fe-S] clusters for insertion into cytoplasmic [Fe-S] proteins (Lange et al., 2001). There is also a requirement for glutathione in the maturation of cytoplasmic proteins, perhaps in the transport of clusters to the cytoplasm, although its precise role has not yet been determined (Sipos et al., 2002). Genetic analysis has shown that all of these proteins play essential roles in yeast. A similar mechanism for cluster assembly appears to exist in humans (Csere et al., 1998; Mitsuhashi et al., 2000) and mutations in the orthologs of the ABC transporter, which result in accumulation of iron in mitochondria, have been shown to be the cause of one form of sideroblastic anemia (Allikmets et al., 1999). In human cells, multiple isoforms of NifS/IscS-, NifU C-terminus, and NifU N-terminus/IscU-like proteins are generated by alternative splicing or the use of alternative start codons and then targeted to either mitochondria or to the cytoplasm or nucleus (Land and Rouault, 1998; Tong and Rouault, 2000; Tong et al., 2003). This suggests that animals have ISC-like systems for [Fe-S] cluster assembly located in distinct subcellular compartments.

One unexpected outcome of genetic studies in a number of these organisms is the identification of additional functions for the NifS/IscS homologs in processes unrelated to [Fe-S] cluster assembly. These include the role of IscS as a sulfur donor for the biosynthesis of thionucleosides in *E. coli, Salmonella enterica*, and humans (Nakai et al., 2001; Lauhon, 2002; Nilsson et al., 2002), the thiazole moiety of thiamine in *E. coli* (Xi et al., 2001), and the molybdopterin cofactor in *Aspergillus* and *Drosophila* (Amrani et al., 2000), as well as in tRNA splicing in yeast (Kolman and Soll, 1993).

NifS, SufS, and SufS-like proteins in a number of organisms have been shown to have both selenocysteine lyase and cysteine desulfurase activity (Mihara and Esaki, 2002). These findings suggest that the NifS/IscS/SufS family of proteins has a broad role in sulfur and selenium utilization within cells.

Despite rapid progress over the past ten years in characterizing the [Fe-S] cluster assembly machinery in eubacteria, yeast, and humans, very little is yet known about this process in plants. There is growing evidence that [Fe-S] cluster biogenesis in plants takes place in both mitochondria and chloroplasts. Takahashi et al. (1991) showed that spinach chloroplasts are able to support the formation of endogenous [Fe-S] clusters targeted for ferredoxin maturation *in vitro*, a finding that was confirmed and extended by Pilon et al. (1995). The first effort to identify proteins that are involved in this process focused on two mercaptopyruvate sulfurtransferase (MST) homologs in *Arabidopsis*, one located in mitochondria, the other in the cytoplasm, which were hypothesized to provide the sulfur for cluster assembly by transferring sulfur ions from mercaptopyruvate to an unidentified acceptor(s) (Nakamura et al., 2000; Papenbrock and Schmidt, 2000). However, a T-DNA knockout mutant for the mitochondrial MST had no apparent effect on [Fe- S] protein activities.

Evidence has recently been described for the presence in chloroplasts of proteins similar to components of the [Fe-S] cluster assembly machinery. Two independent studies characterized an *Arabidopsis* gene with homology to the *nifS/iscS/sufS* family (Léon et al., 2002; Pilon-Smits et al., 2002). Phylogenetic analysis of the predicted gene product, designated AtNFS2 (or ATCpNIFS), places it in a heterogeneous group of proteins that are most closely related to SufS (group II) (Tachezy et al., 2001). The

chloroplast location of AtNFS2 has been confirmed experimentally using an *in vitro* chloroplast import assay and by GFP localization in protoplasts (Léon et al., 2002; Pilon-Smits et al., 2002). Moreover, AtNFS2 has a much higher desulfurase activity with selenocysteine than cysteine, similar to *E. coli* SufS (Pilon-Smits et al., 2002). [Fe-S] cluster assembly by AtNFS2 has not yet been demonstrated; however, the similarity of this protein in sequence and properties to SufS suggests that it could have similar functions, i.e., in [Fe-S] cluster assembly as well as selenoprotein synthesis. Relevant to the former, potential scaffold proteins for the assembly of clusters in chloroplasts have recently been characterized (Léon et al., 2003). *Arabidopsis* contains five proteins with homology to the C terminus of *A. vinelandii* NifU; three of these proteins, AtNFU1-3, form a separate class that is most closely related to *Synechosystis* PCC6803 NFU, including a unique C-terminal extension, and is targeted exclusively to chloroplasts. The genes are differentially expressed and could provide the partner proteins for assembly of [Fe-S] clusters by AtNFS2 in the chloroplasts of different tissues.

There is compelling evidence that plants also contain a mitochondrial system for [Fe-S] cluster biogenesis, similar to yeast and animals. Kushnir et al. (2001) identified three genes in *Arabidopsis* with homology to yeast *ATM1p*, which encodes the ABC transporter involved in export of Fe-S clusters from mitochondria in yeast. One of these genes, *Starik1,* has been shown to complement a defect in *ATM1p* and to support maturation of [Fe-S] proteins in the yeast cytoplasm. The *sta1* mutation results in dwarfism and chlorosis, as well as a small increase in Fe accumulation in mitochondria. *sta1* mutant plants also exhibit elevated expression of two other homologs of yeast genes required for maturation of [Fe-S] proteins, as well as genes involved in the

response to oxidative stress and DNA damage. However, no significant effects were observed on activities of the [Fe-S] enzymes, aconitase and ferrochelatase, perhaps because AtSTA2 and AtSTA3 are functionally redundant with AtSTA1.

Arabidopsis mitochondria also appear to contain numerous proteins with homology to integral components of the Nif/Isc/Suf machinery. These include AtNFS1, a protein that is closely related to group I NifS, IscS, and mitochondrial cysteine desulfurase enzymes (Kushnir et al., 2001; Tachezy et al., 2001; Pilon-Smits et al., 2002); AtNFU4 and AtNFU5, which have strong similarity to the bacterial, yeast, and human NifU C-terminus class of proteins, (Léon et al., 2003); and AtISU1 and AtISU3, which have homology to the NifU N-terminus/IscU (unpublished data). The N-terminal sequences of AtNFS1 and AtNFU4 have been shown to target GFP to mitochondria in tobacco cells and *Arabidopsis* protoplasts, respectively (Kushnir et al., 2001; Léon et al., 2003). However, nothing more is known about the cluster assembly system in plant mitochondria. Here we describe the detailed characterization AtNFS1, including direct evidence that this protein can function in the *in vitro* assembly of [Fe-S] clusters, as well as genetic evidence for the importance of AtNFS1 in fundamental metabolic processes.

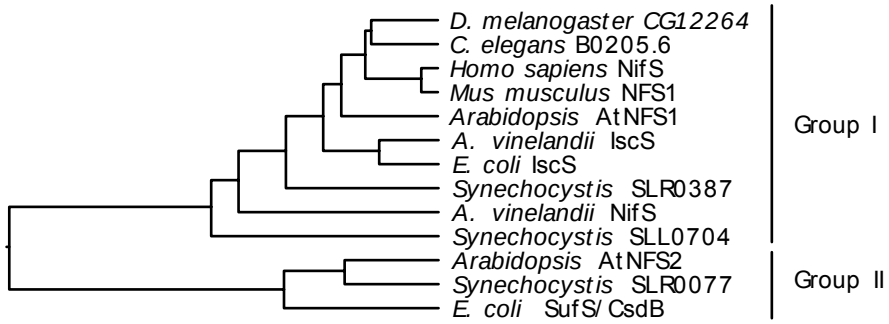
RESULTS

Characterization of the *AtNFS1* gene

Arabidopsis contains a single gene, *AtNFS1* (At5g65720), that has high sequence similarity with the group I *iscS/nifS*-like genes (Kushnir et al., 2001; Tachezy et al., 2001; Léon et al., 2002) (Figure 1A). *AtNFS1* does not contain introns, similar to many of the group I genes in eukaryotes, including mammals and amitochondriate

Figure 1. Phylogenetic analysis of the AtNFS1 protein. (A) Rooted tree showing relationships among NifS/IscS proteins from *A. vinelandii, Arabidopsis, E. coli, Homo sapiens, Mus musculus,* and *Synechocystis* PCC6803, and the distinction between members of Group I and Group II. **(B)** Alignment of Group I sequences from A, with identical residues in black boxes. Residues involved in substrate binding are indicated with triangles, with an open triangle for the active site cysteine. Residues involved in PLP binding are indicated by circles. The histidine involved in substrate deprotonation is indicated with a diamond. C terminal sequences involved in interaction with IscU are bracketed.

A.



B.


AtNFS1
Homo sapiens NFS1
Mus musculus NFS1
A. vinelandii IscS
E. coli IscS

eukaryotes. In contrast, the homologous genes from *Drosophila melanogaster* and *Caenorhabidis elegans* contain one and nine introns, respectively, while the *Arabidopsis* group II gene, *AtNFS2*, contains eight introns (Léon et al., 2002; Pilon-Smits et al., 2002) (GenBank; data not shown). The protein predicted from *AtNFS1* exhibits 54.7% overall amino acid identity with *A. vinelandii* IscS, with absolute conservation of residues involved in substrate and PLP cofactor binding as well as the region surrounding the active site cysteine (Zheng et al., 1993; Zheng et al., 1994; Kaiser et al., 2000; Kurihara et al., 2003). C terminal residues involved in interaction of *A. vinelandii* IscS with IscU (Urbina et al., 2001) are also highly conserved in the *Arabidopsis* protein, with 69% identity (Figure 1B); it is noteworthy that similar sequences are not present in AtNFS2 (Léon et al., 2002). AtNFS1 has an amino terminal extension relative to the bacterial proteins that, based on PSORT and TargetP predictions, is likely to direct the protein to mitochondria. Analysis of the EST database indicates that *AtNFS1* is expressed at high levels in a variety of tissues, including cold-treated rosettes, flower buds, and green siliques.

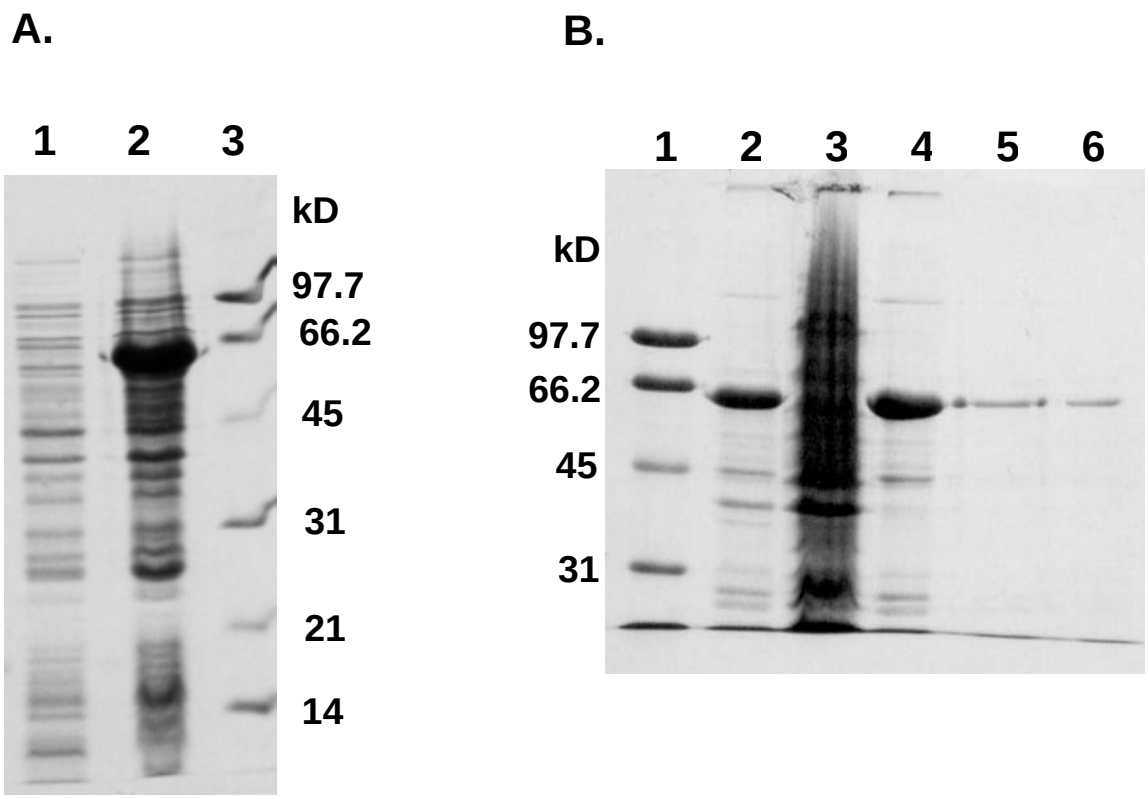
Biochemical Characterization of recombinant AtNFS1

To obtain protein for biochemical analysis, the *AtNFS1* coding region was inserted into pET32a, allowing production of the *Arabidopsis* protein fused to the C terminus of thioredoxin (TRX-AtNFS1). Use of the *E.coli* strain, BL21-CodonPlus-RIL, significantly improved expression efficiency, most likely because 18 arginine residues in AtNFS1 are specified by codons that are rarely used in *E. coli.* When these cells were grown to mid-log phase in LB medium containing 10 μM PLP and then induced with 1%

lactose, accumulation of a protein of approximately the predicted size (50.3 kD from AtNFS1 plus 17.1 kD from TRX and linker sequences) was observed (Figure 2A). However, this protein was found primarily in the insoluble fraction (Figure 2B, lanes 2 and 3). Several protocols were tested for purification of the recombinant protein, including solubilization in 2% SDS followed by refolding. A variety of growth temperatures, growth media, vectors, host cells, and extraction buffers were also used in an effort to enhance production of a soluble form of the protein. In all cases the TRX-AtNFS1 protein was either not expressed, was inactive based on the cysteine desulfurase activity assay described below, and/or aggregated during the refolding step. A protocol was therefore developed involving solubilization in 8M urea, followed by dilution to 0.2 M urea to allow refolding and then dialysis in Tris buffer and concentration. The purification process was monitored by SDS-PAGE and resulted in a faint but substantially pure band (Figure 2B). N-terminal sequencing identified five residues from TRX, confirming that the desired protein had been purified.

Using this method only about 5% of the total TRX-AtNFS1 protein was recovered, but this protein was in an active form. In a kinetic assay for cysteine desulfurase activity (Zheng et al., 1993), purified TRX-AtNFS1 catalyzed the release of 12 nmol of sulfide min^{-1} (mg of pure protein)$^{-1}$ from L-cysteine. This compares with 89.4 nmol of sulfide min^{-1} for the *A. vinelandii* NifS (Zheng et al., 1993), 78 nmol of sulfide min^{-1} for the *E. coli* NifS (Flint, 1996), 67.6 nmol of sulfide min^{-1} for *A. vinelandii* IscS (Zheng et al., 1998), and 9.96 nmol of sulfide min^{-1} for *Helicobacter pylori* NifS (Olson et al., 2000).

Figure 2. Expression and purification of recombinant AtNFS1. (A) Expression of TRX-AtNFS1 in *E. coli* BL21(DE3) RIL cells, before and after induction with 1% lactose. Lane 1, total protein before induction; lane 2, total protein 4 h after induction; lane 3, molecular weight marker (low range SDS-PAGE standards; BioRad). **(B)** TRX-AtNFS1 purification. Lane 1, molecular weight marker; lane 2, pellet before 8 M urea; lane 3, first supernatant before 8 M urea; lane 4, pellet after 8 M urea; lane 5, supernatant after 8 M urea; lane 6, fraction concentrated by ultrafiltration. Each lane was loaded with 10 µl of sample.



The ability of the recombinant AtNFS1 protein to mediate *in vitro* assembly of [Fe-S] clusters was tested using the method of Yuvaniyama et al. (2000a). Reactions were performed using 0.8 μM TRX-AtNFS1 in conjunction with 28 μM of either AtISU1, an IscU homolog from *Arabidopsis* (A. Frazzon, Warek, and Winkel, unpublished results), or *A. vinelandii* IscU. This experiment demonstrated the ability of TRX-AtNFS1 to mediate the time-dependent assembly of a chromophoric species in AtISU1 and IscU that exhibits the characteristic absorbance inflections of [2Fe-2S] clusters at 325 nm and at 420 (Figure 3). Significantly higher activity was observed at pH 8.5 than at pH 7.4, the pH optimum for *in vitro* cluster assembly by bacterial NifS/IscS proteins (data not shown).

Subcellular Localization of AtNFS1

The full coding region of *AtNFS1* was fused to the N terminus of the plant-optimized reporter gene, *mGFP5*, and expressed in transgenic *Arabidopsis* plants under the control of the constitutive double-enhanced CaMV 35S promoter. None of the transformants exhibited any notable phenotypes relative to wild type. Leaves from five independent T1 lines as well as two homozygous and one heterozygous T4 line were examined by confocal laser scanning microscopy. In all of these lines, AtNFS1::GFP was targeted specifically to mitochondria (Figure 4), while GFP expressed in the absence of AtNFS1 was localized exclusively to the cytoplasm (not shown). Mitochondrial localization of AtNFS1 is consistent with predictions from PSORT and Target P analysis and also with a previous report showing that the N-terminal 54 amino

Figure 3. Assembly of Fe-S clusters by *Arabidopsis* AtNFS1. Cluster biosynthesis was performed in a quartz cuvette under anaerobic conditions in a reaction containing 0.8 μM AtNFS1 and 30 μM IscU (B) or AtISU1 (C). Assembly was monitored as a change in absorbance at 280-800 nm before (A) or 180 min after (B and C) addition of 1 mM L-cysteine.

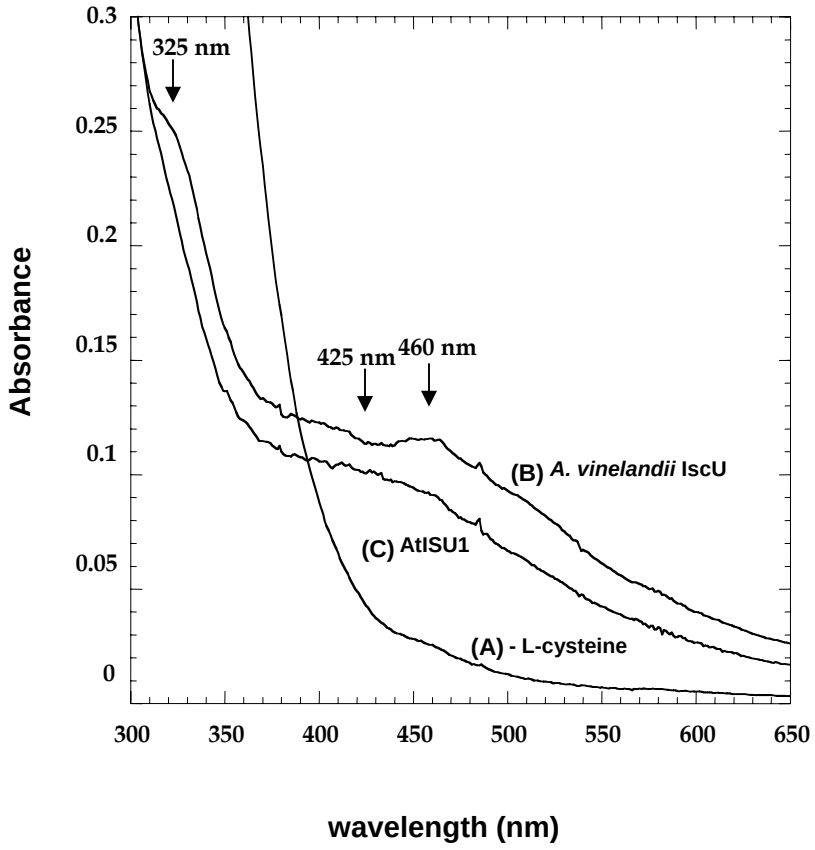
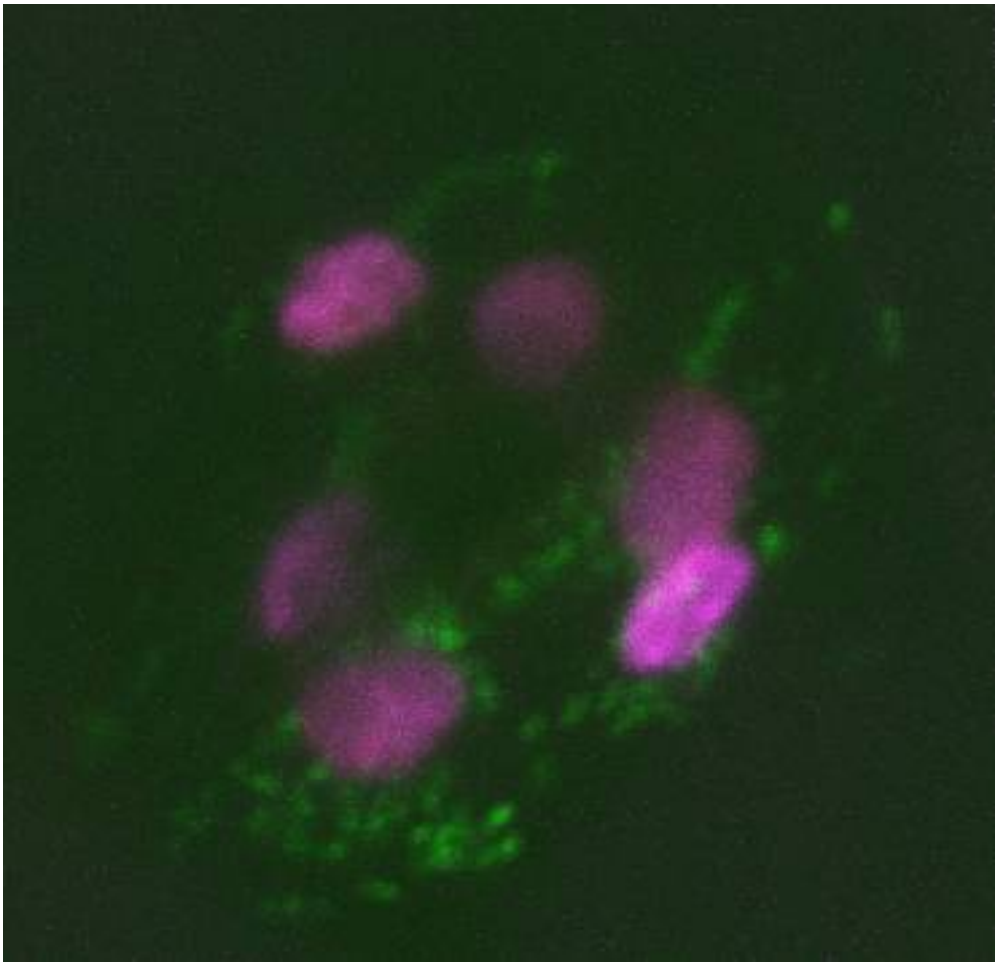


Figure 4. Localization of AtNFS1 in *Arabidopsis* leaves. *AtNFS1* was fused to the N terminus of the plant-optimized reporter gene, *mGFP5*, and expressed in transgenic *Arabidopsis* plants under the control of the constitutive double-enhanced CaMV 35S promoter. Leaves from 4-week-old plants were examined by confocal laser scanning microscopy. GFP fluorescence is shown in green, autofluorescence from chloroplasts is in red.



acid residues of this protein direct GFP to mitochondria in tobacco cells (Kushnir et al., 2001).

Phenotypic Effects of Altered AtNFS1 expression in transgenic plants

In an effort to correlate AtNFS1 activity with specific physiological processes, transgenic plants were generated in which the *AtNFS1* coding region was placed in the forward (sense) or reverse (antisense) orientation behind the double-enhanced 35S promoter. Multiple independent T1 plants were recovered, of which six containing the sense construct and three containing the antisense construct were isolated as homozygotes in the T3 generation. No overt phenotypes were observed for plants overexpressing *AtNFS1* in the sense orientation, similar to what was found with the AtNFS::GFP construct, although several lines appeared to be somewhat larger than the wild type (not shown). In contrast, plants expressing *AtNFS1* in the anti-sense orientation exhibited a number of striking visible phenotypes. Several lines developed chlorotic spots on the leaves, as shown in Figure 5. Many of these plants also exhibited developmental abnormalities, including scalloped edges on new leaves, disorganized inflorescences, and a noticeable increase in axillary shoot development at the axils of both rosette and cauline leaves, resulting in a bushy phenotype. These plants were also noticeably shorter than the wild-type counterpart. Interestingly, with the exception of the influorescence phenotype, these developmental effects are similar to those observed for the *axr1* mutant, which appears to carry a defect in auxin signaling (Greb et al., 2003). Chlorosis and stunted growth were also observed in the *Arabidopsis starik*

Figure 5. Phenotypes of plants expressing *AtNFS1* in the antisense orientation.

A, C, D, F, G, H, I, transgenic plants; B, E, wild type.

mutant, carrying a disruption of a mitochondrial ABC transporter implicated in export of [Fe-S] clusters (Kushnir et al., 2001).

Mutations that disrupt the assembly of [Fe-S] clusters in yeast and humans result in the accumulation of high levels of iron in mitochondria (Allikmets et al., 1999; Garland et al., 1999; Lill and Kispal, 2000). In contrast, no appreciable differences were found in the total iron content of AtNFS1 antisense plants relative to non-transformed plants (data not shown). This could reflect the fact that iron levels in mitochondria represent only a small fraction of the total iron content. However, similar result was obtained for the *starik* mutant, which exhibited no detectable difference in total iron levels and only a 1.5- to 1.8-fold higher level in mitochondria of achlorophyllous callus tissue (Kushnir et al., 2001). This suggests that plants have mechanisms for mediating mitochondrial iron homeostasis that are not present in other eukaryotes.

The antisense lines were also characterized with respect to photosynthetic capacity. Chlorophyll fluorescence measurements were performed on single leaves from 4 to 6 plants for each of the three homozygous antisense line and 5 to 8 plants of the wild type under two different illumination conditions, favoring either photosystem I (PSI) or photosystem II (PSII) activity. Chlorotic spots were not present on the leaves of the transgenic plants at the time the experiment was performed. The F_v/F_m ratios, an indicator of the physiological state of the photosynthetic apparatus in intact plant tissues, were between 0.85 and 0.79 for all of the plants (Table I). These values are typical for dark-acclimated, healthy, unstressed plants (Maxwell and Johnson, 2000). Values were then determined for the quantum yield ($\phi_{PSI/PSII}$), which measures the proportion of absorbed light that is used in photochemistry; photochemical quenching

Table I. Photosynthetic efficiency of PSII in AtNFS1 sense and antisense plants.

Fv/Fm, quantum yield (ϕ_{PSII}), photosynthetic quenching (qP) and nonphotosynthetic quenching (NPQ) were determined from chlorophyll fluorescence measurements made in red light, favoring PSI, or white light, favoring PSII, as described in the Materials and Methods. Average values for single leaves from 5-10 individual plants are given (numbers in parentheses indicate the number of plants tested for each line), together with standard errors. Asterisks indicate values that differ from the wild type control with a p value ≤ 0.05.

	Fv/Fm	**$\phi_{PSI/PSII}$**	**qP**	**NPQ**
Red light (PSI)				
WT (5)	0.810 ± 0.009	0.532 ± 0.052	0.701 ± 0.060	0.299 ± 0.055
3.11 (4)	0.802 ± 0.010	0.487 ± 0.045	0.657 ± 0.051	0.286 ± 0.027
3.12 (6)	0.790 ± 0.031	0.445 ± 0.031	0.621 ± 0.037	0.385 ± 0.082
3.17 (5)	0.798 ± 0.003	0.478 ± 0.019	0.636 ± 0.021	0.208 ± 0.040
White light (PSII)				
WT (8)	0.832 ± 0.007	0.617 ± 0.063	0.823 ± 0.055	0.466 ± 0.139
3.11 (5)	0.839 ± 0.004	0.728 ± 0.009*	0.904 ± 0.012*	0.149 ± 0.024*
3.12 (5)	0.843 ± 0.003	0.695 ± 0.022*	0.862 ± 0.022	0.210 ± 0.076*
3.17 (5)	0.840 ± 0.013	0.643 ± 0.050	0.805 ± 0.070	0.304 ± 0.089*

(qP), an indication of the proportion of PSII reaction centers that are open; and nonphotosynthetic quenching (NPQ), which is linearly related to heat dissipation. Little or no effect on PSI function was observed in any of these lines (Table I). There was also little or no effect on photochemical quenching for PSII. However, all three lines exhibited a marked decrease in nonphotochemical quenching for PSII; consistent with this, two of the three lines also showed a small, but statistically significant, increase in quantum yield for PSII. This suggests that the photosynthetic efficiency of PSII was actually higher than for wild type and that this was due to a decrease in the dissipation of energy as heat. A possible explanation for this surprising result is that perturbations in the mitochondrial [Fe-S] cluster assembly machinery, which are likely to negatively impact processes such as respiration, might result in a compensatory increase in photosynthetic efficiency.

DISCUSSION

[Fe-S] clusters are ancient and ubiquitous structures that have well-defined roles in a wide range of fundamental cellular processes ranging from electron transport in energy-generating systems to catalysis, transcriptional regulation, and sensing of environmental conditions. It is therefore perhaps not surprising that the biogenesis of these clusters is also largely conserved, with only a few exceptions. The present study provides direct evidence for a plant protein with similarity to *nif/isc* systems that functions in mitochondria, as in other eukaryotes, in the assembly of [Fe-S] clusters. When expressed as a thioredoxin fusion in *E. coli*, AtNFS1 exhibits cysteine desulfurase activity and is able to mediate the *in vitro* assembly of transient [Fe-S] clusters in either

Arabidopsis AtISU1 or *A. vinelandii* IscU. GFP fusion experiments localized AtNFS1 exclusively to mitochondria, consistent with phylogenetic analysis and predictions based on the N-terminal sequence, as well as previous evidence that the N terminus of this protein targets GFP to mitochondria in tobacco cells (Kushnir et al., 2001).

The mitochondrial machinery in which AtNFS1 operates may be in addition to a distinct chloroplast-localized assembly system that has similarity to the bacterial *suf* machinery and is mediated, in part, by AtNFS2. It is interesting to speculate that the suf-like system from cyanobacteria survived genome reduction during plant chloroplast evolution, while the nif/isc-like system from a eubacterium evolved into the assembly machinery found in mitochondria of plants and other eukaryotes. This possibility is supported by phylogenetic analysis of the corresponding protein sequences (Fig. 1A and Tachezy et al., 2001). It is interesting to note, however, that although AtNFS1 is more closely related to *A. vinelandii* NifS in sequence, the pH optimum of this enzyme is more like that of the cyanobacterial NifS-like protein (Jaschkowitz and Seidler, 2000; Yuvaniyama et al., 2000b).

Antisense expression of *AtNFS1* induced a number of striking developmental phenotypes in transgenic plants, including scalloped edges on new leaves, disorganized inflorescences, and a noticeable increase in axillary shoot development. These plants also exhibited chlorotic spots on leaves, similar to what was reported for the *sta1* mutant deficient in an ABC transporter involved in export of clusters from mitochondria (Kushnir et al., 2001). These phenotypes suggest a role for AtNFS1 in the maturation of [Fe-S] proteins involved in hormone biosynthesis and in the response to photo-oxidative stress, as suggested for *sta,* and in sulfur assimilation. Interestingly, both antisense

expression and ectopic expression of *AtNFS1* appeared to increase the efficiency of PSII function, suggesting that tight regulation of *AtNFS1* expression may be an important factor in general sulfur trafficking and iron homeostasis in plants and that alterations in the mitochondrial system impact, either directly or indirectly, chloroplast function. A survey of the EST database indicates that *AtNFS1* is expressed at high levels in a variety of tissues, similar to *AtNFS2* (Kushnir et al., 2001; Léon et al., 2002), further pointing to a broad role for this gene in iron and sulfur trafficking in plants.

There is no evidence for localization of either of the plant nifS/iscS/sufS-like proteins in the cytoplasm or nucleus, as has been reported in humans (Tong et al., 2003), although very low levels would not have been detected in the experiments performed to date. *Arabidopsis* does contain a cytoplasmic protein, defined by the *ABA3* locus, that contains a NifS-like sulfurtransferase domain together with a second domain that may be involved in recognizing two target enzymes, aldehyde oxidase and xanthine dehydrogenase, that are activated by sulfuration (Bittner et al., 2001). However, a role in [Fe-S] cluster assembly has not been demonstrated for ABA3. No other NifS/IscS/SufS-like proteins appear to be encoded by the *Arabidopsis* genome, including a third class defined by the C-DES cyst(e)ine desulfurases of the cynanobacteria, *Synechocystis* and *Thermosynechococcus elongates* (Leibrecht and Kessler, 1997; Nakamura et al., 2002). Thus the [Fe-S] cluster assembly machinery in plants may very well be restricted to mitochondria and chloroplasts.

The complements of apoproteins that are targeted by the two assembly systems remain to be identified; for the mitochondrial machinery these presumably include mitochondrial [Fe-S] proteins and all or a subset of cytoplasmic [Fe-S] proteins, but not

those destined for the chloroplast. In yeast, absence of the transporter Atm1p affects only cytoplasmic [Fe-S] proteins, indicating that maturation of mitochondrial [Fe-S] proteins occurs following transport into the organelle (Kispal et al., 1999). This type of analysis is confounded in *Arabidopsis* due to the presence of redundant transporters in this organism (Kushnir et al., 2001). However, the absence of effects on PSI in the *AtNFS1* antisense plants supports the notion that maturation of chloroplast [Fe-S] proteins occurs independently of the mitochondrial system. At the same time, there is cytological evidence for physical contacts between mitochondria and chloroplasts (Köhler et al., 1997) and there are several precedents for effects of mitochondrial mutations on chloroplast structure and function, although the molecular basis is also not well understood in any of these cases (Roussell et al., 1991; Gu et al., 1993; Sakamoto et al., 1996; Abdelnoor et al., 2003). The phenomenon of light-enhanced dark respiration, in which respiratory O_2 uptake in darkness increases rapidly upon illumination The enhanced efficiency of PSII in *AtNFS1* antisense plants was surprising and suggests that chloroplasts may have the ability to compensate for deficiencies in mitochodrial function such as respiration. At the same time, the appearance of chlorotic spots on the leaves of these plants could indicate that these plants are more sensitive to photoinhibition, protection from which has been linked to mitochondrial respiration (reviewed in Padmasree et al., 2002). Further characterization of the AtNFS1 down-regulated plants could thus promises to not only provide additional information on the mitchondrial [Fe-S] cluster biogenesis machinery per se, but new insights into the inter-dependence of the two organelles.

The *Arabidopsis* genome contains over 40 genes with predicted protein products

homologous to components of the [Fe-S] cluster biosynthesis machinery in other organisms, although very little is yet known about any of them. These include three genes with homology to the NifU N-terminus/IscU (AtISU1-3; 66-67% amino acid identity), five genes with homology to the NifU C terminus (AtNFU1-5; 17-22% identity), and four genes with homology to IscA (AtISA1-4; 30-40% identity), all of which could function as molecular scaffolds for [Fe-S] assembly. Three of the AtNFU proteins (Léon et al., 2003) and at least two of the AtISU proteins (Warek and Winkel, unpublished data) appear to be located in mitochondria and one of these, AtISU1, has now been shown to function as a scaffold for *in vitro* cluster assembly with AtNFS1. However, it is not yet known which of these proteins serve as the true *in vivo* partner for cluster assembly by AtNFS1. Identifying the specific components of the mitochondrial and chloroplast NifS/IscS/SufS systems in plants, characterizing the potentially diverse functions of each these proteins, and defining the relationships between the two systems represent the immediate challenges to understanding how this machinery has co-evolved in two distinct subcellular locations to serve the unique requirements of the plant cell.

MATERIALS AND METHODS

Expression and Purification of Recombinant AtNFS1 and AtISU1 in *E. coli*

The *AtNFS1* (At5g65720) coding region (1362 bp including the stop codon, no introns), was amplified by polymerase chain reaction (PCR) from bacterial artificial chromosome (BAC) clone F6H11 (Arabidopsis Biological Resource Center) for expression in *E. coli*. The reaction was performed using *Pfu* DNA polymerase

(Promega) and forward and reverse primers, 5'-CATGCC**ATG**GCGTCTAAGGTAATC-3' and 5'-ATCGGATCCGTTGAA**TCA**GTGTTG-3' (nucleotides complementary to *AtNFS1* underlined, start and stop codons in bold), that added *Nco*I and *Bam*HI restriction sites, respectively, to allow directional cloning into the pET32a expression vector (Novagen). The 3' segment of the *AtISU1* (At4g22220) coding region that has homology to *A. vinelandii IscU* (408 nucleotides including the stop codon, corresponding to amino acids 27-167) was amplified from expressed sequence tag (EST) clone, K3F7TP. These sequences were amplified using the forward primer, 5'-CATGCAT**ATG**CGAACCTACC-3', and reverse primer 5'-GATCGGATCCGAA**TCA**AGCCTGTG-3', which added *Nde*I and *Bam*HI sites, respectively, to allow directional cloning into the pET16b (Novagen). Inserts were sequenced on both strands using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

Sequence analysis and phylogenetic tree construction were performed using the Lasergene suite of DNA analysis programs (DNASTAR, Madison, WI). Protein sequence comparisons were made using the following: *A. vinelandii* IscS (accession number T44281), *A. vinelandii* NifS (P05341), *Arabidopsis* AtNFS1 (O49543), *Arabidopsis* AtNFS2 (AAF22900), *Caenorhabditis elegans* B0205.6 (AAC16992), *Drosophila melanogaster* GC12264 (AAF53143), *E. coli* IscS (P39171), *E. coli* SufS/CsdB (BAB21542), *Homo sapiens* NifS (AAD09187), *Mus musculus* NFS1 (Q9Z1J3), *Synechocystis* PCC 6803 SLR0077 (Q55793), *Synechocystis* PCC 6803 SLR0387 (NP 442039), and *Synechocystis* PCC 6803 SLL0704 (BAA16678). Analysis

of targeting sequences was performed using PSORT (http://psort.nibb.ac.jp/) and Target P (http://www.cbs.dtu.dk/services/TargetP/).

The expression construct, pET32a-AtNFS1, was transformed into *E. coli* strain BL21-CodonPlus-RIL (Stratagene). Cells were grown to mid-log-phase at 37°C in 500 ml LB-$amp_{100}cm_{34}$. AtNFS1 production was induced when cells reached ~160-180 Klett units by the addition of lactose to 1% final concentration. The cells were allowed to continue growing overnight at room temperature, harvested by centrifugation, and stored at -80^0C. For purification of AtNFS1, 10 g of cell paste was resuspended in 25 mM Tris, pH 7.4 at 2.5 ml per g of cells. After cell disruption by sonication, the insoluble material was removed by centrifugation at 10,000 x g for 20 min. The protein was purified from inclusion bodies by resuspending the pellet in 8 M urea, 20 mM Tris, pH 7.4 at 1 ml per g of cell paste followed by incubation for 1 h at room temperature. After centrifugation at 18,000 x g for 15 min, the supernatant was diluted with 0.5 M NaCl, 0.5 mM PLP, 20% glycerol, and 20 mM Tris, pH 8.0 to bring the urea to a concentration of 0.2 M. The solution was incubated at 4°C for 1 h and the precipitate removed by centrifugation at 18,000 x g for 15 min. The supernatant was dialyzed at 4°C overnight against 20 mM Tris, pH 8.0, concentrated to 15 ml using an Amicon ultrafiltration device fitted with a YM30 filter, and dialyzed overnight against 25 mM Tris, pH 7.4. The purified recombinant AtNFS1 protein was frozen in liquid nitrogen and stored at -80 °C. The protein concentration was determined by the biuret method (Gornall et al., 1949) and purity was assessed by SDS-PAGE. Approximately 10 mg of approximately 90% pure AtNFS1 was obtained using this procedure. N-terminal sequencing was performed at the University of Virginia Biomolecular Research Facility.

The expression plasmid, pET16b-AtISU1, was transformed into *E.coli* BL21(DE3)pLys cells (Novagen). The cells were grown at 37°C in 500 ml LB-$amp_{100}cm_{34}$. AtISU1 production was induced as described above and the cells were then cultured for an additional 4 h at room temperature, harvested by centrifugation and stored at -80°C. For purification of ISU1, 20 g of cell paste were resuspended in 50 ml of 25 mM TrisHCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole, containing 1 mM phenylmethylsulfonyl fluoride. After cell disruption by sonication, the insoluble material was removed by centrifugation at 10,000 x g for 20 min. The supernatant was loaded on a 1 cm x 15 cm column packed with Q-Chelation Sepharose (Pharmacia) and the protein eluted in 25 mM TrisHCL, pH 7.9, 0.5 M NaCl, 500 mM imidazole. The sample was then immediately loaded on a 3 cm x 30 cm column packed with S-300 resin (Pharmacia) and equilibrated in 25 mM TrisHCl, pH 7.4, 0.5 M NaCl in order to remove the imidazole from the sample. The fraction containing the protein, based on absorbance at 280 nm, was concentrated to 10 ml using an Amicon ultrafiltration device fitted with a YM10 filter and then frozen in liquid nitrogen and stored at -80°C. This procedure yielded approximately 30 mg of ISU1 that was estimated to be 90% pure, based on analysis on a Coomassie-stained SDS polyacrylamide gel. IscU protein was purified from *A. vinelandii.*

Activity Assays

Cysteine desulfurase activity was assayed as described in Zheng et al. (1993) using 40 µg of purified recombinant protein in a 1 ml reaction. *In vitro* assembly of Fe-S clusters was carried out essentially as described by Yuvaniyama et al. (2000a). The

reactions were performed under anaerobic conditions in a quartz cuvette and contained 28 µM AtISU1, 0.8 µM AtNFS1, 2.5 mM ß-mercaptoethanol, 0.1 mM ferric ammonium citrate, 2 mM L-cysteine, and 20 mM Tris.HCl pH 8.5 in 1 ml total volume. Assembly was monitored by UV-visible spectroscopy over a range of 280-600 nm before and 180 min after the addition of L-cysteine. Positive and negative control reactions were performed using IscU from *Azotobacter vinelandii* and in the absence of L-cysteine, respectively.

AtNFS1 **Constructs for Expression in Plants as mGFP5 Fusions and in the Sense and Antisense orientation.**

To prepare fusion constructs of AtNFS1 with GFP, F6H11 was again used as a template with *Pfu* polymerase to amplify both the 453 aa *AtNFS1* coding region, this time without the stop codon, and sequences encoding the amino-terminal 59 aa. The same forward primer was used as above, adding a *NcoI* site at the 5' end of the coding region, together with either 5'-CATGCCATGGCGTGTTGAGACCATTGAATG-3' or 5'-CATGCCATGGCATATCTAAGTAAAGAGGTC-3' as the reverse primer, adding a *NcoI* site at the 3' end. The resulting fragments were digested with *Nco*I and cloned in-frame with the amino terminus of mGFP5 in pAVA393 (von Arnim et al., 1998). The 35S::AtNFS1$_{1\text{-}453}$-GFP and 35S::AtNFS1$_{1\text{-}59}$-GFP fusions, including the double-enhanced cauliflower mosaic virus (CaMV) 35S promoter, Tobacco Etch Virus (TEV) translational leader, and nopaline synthase (nos) terminator sequences, were excised with *Sal*I and *Xma*I and cloned into the corresponding sites in the binary vector, pBIB (Becker, 1990). The integrity of the constructs was tested by examining GFP activity by confocal laser

scanning microscopy following particle bombardment into onion epidermal cells (Scott et al., 1999).

Sense and antisense constructs for *AtNFS1* were assembled using sequences from the pRTL2 vector (Carrington and Freed, 1990). A cassette containing the same double-enhanced CaMV 35S promoter, TEV translational leader, and nos terminator sequences as in pAVA393 was excised from this plasmid with *Hin*dIII and inserted into pBluescript (Stratagene) to acquire polylinker sequences that facilitated subsequent cloning steps. The *AtNFS1* coding region, including the stop codon, was again amplified from F6H11using *Pfu* polymerase, the same forward primer as above, and a reverse primer that added a *NcoI* site but this time included the stop codon, 5'-CATGCCATGG**TCA**GTGTTGAGACCATTG-3'. The resulting fragment was digested with *Nco*I and cloned into the corresponding site in pBLRTL2. Orientation of the inserts was determined by PCR and restriction enzyme analysis. The resulting constructs, including the promoter and terminator sequences, were excised with *Sal*I and *Kpn*I and cloned into the corresponding sites in pBIB.

Plants and Growth Conditions

Arabidopsis thaliana ecotype Columbia was used in these studies. Seeds were plated as described previously (Kubasek et al., 1992). Briefly, the seeds were surface sterilized by soaking in 100% ethanol for 1 min followed by 50% of 1.5% NaOCl, 0.5% Triton X-100 for 5 min, followed by three rinses in sterile distilled water. The seeds were then suspended in 2 ml 0.15% agar and spread on Murashige Skoog (MS) medium, pH 5.7, containing 2% sucrose and 0.8% agar. The plates were sealed with

Nescofilm® (Karlan), wrapped in aluminum foil, and the seeds were vernalized for 2-4 d at 4°C. The plates were then incubated at 22°C under continuous light for approximately 10 days. The seedlings were transferred to soil and grown at 22°C under a 16 h/8 h light/dark cycle. Transgenic plants were selected on MS-sucrose plates supplemented with kanamycin.

Plant Transformation

All constructs in the pBIB and pCAMBIA1302 vectors were used to transform *Agrobacterium tumefaciens* strain GV3101 by the freeze-thaw method (Chen et al., 1994). *Arabidopsis* ecotype Columbia plants were transformed using the floral dip method followed by vacuum infiltration (Clough and Bent, 1998). Seeds were harvested and first generation (T_1) seedlings transformed with pBIB-derived constructs were selected on MS-sucrose agar medium containing 100 µg/ml kanamycin. The plates were vernalized in darkness at 4°C for 2 d and then transferred to constant white light at 22°C. After 10 d, green seedlings were transferred to soil and grown at 22°C under a 16 h/8 h light/dark cycle. Second generation (T_2), third generation (T_3), and fourth generation (T_4) seeds were collected and selected on MS sucrose plates containing kanamycin to follow segregation of the transgene and identify homozygous lines. Resistant plants were transferred to soil for propagation.

Microscopy

For CLSM microscopy, fully expanded rosette leaves from 3-4 week old plants were mounted in water under cover slips. Specimens were examined with an 40X C-

Apochromat objective lens (water corrected, numerical aperture (N.A.) using a LSM 510 Confocal Laser Scanning Microscope (Carl Zeiss Inc., Thornwood, NY). Fluorescent images were obtained up to 532 X 512 pixel resolution by using the 488 nm excitation line of an argon laser for GFP fluorescence and a helium-neon laser (633 nm) for chlorophyll autofluorescence, with 505 to 530 nm band pass (BP) and 650 nm long pass (LP) filters for GFP and chlorophyll, respectively. Autofluorescence in wild-type tissues were used to set the threshold level above which GFP fluorescence was detected in the transgenic lines. Samples were optically sectioned up to 1 μm to reconstruct 3-D images using the software package supplied with the microscope. Differential interference contrast (DIC) reference images were also collected. Images were exported as JPEG files and further processed with Adobe Photoshop (version 5.02; Adobe Systems, San Jose, CA).

Phenotypic Analysis and Measurement of Photosynthetic Capacity

The determination of iron content in dry leaves from several of the transgenic and wild type plants was performed by the laboratory of Michael Grusak (USDA-ARS Children's Nutrition Research Center) using atomic absorption as described previously (Marentes and Grusak, 1998).

Chlorophyll fluorescence was measured in wild-type and transgenic *Arabidopsis* plants using light conditions adapted from Pfannschmidt et al. (1999). Seedlings were germinated on MS medium either with (transgenic) or without (wild type) kanamycin as described above, then transferred to soil. After 7 weeks half the plants were grown for at least 7 more days under white fluorescent light (favoring PSII) and the other half

under white fluorescent light with a red filter (Lee Filters, 027 Medium red) (favoring PSI). Chlorophyll fluorescence was measured *in vivo* using an OS-500 pulse amplitude modulated fluorometer (Opti-Sciences, Tyngsboro, MA, USA). One leaf on each plant was adapted to darkness for 15 min. The measuring beam was then turned on and minimal fluorescence (F_o) was measured. Leaves were next exposed to a 500 ms saturating flash (6000 µmol $m^{-2}s^{-1}$) to determine maximal fluorescence (F_m) and the $F_v/F_m = (F_m-F_s)/F_m$ ratio was calculated. Leaves were then illuminated with actinic light (100 µmol m^{-2} s^{-1} at 670 nm). Once instantaneous fluorescence returned to the level of F_o, another saturating pulse was fired. The actinic light was then turned off and the leaf was illuminated with far-red light (735 nm) to calculate F_{od}, the possible quenching of F_o. The effective quantum yield of PSII was calculated as $Y= (F_{ms}-F_s)/F_{ms}$, photochemical quenching was calculated as $qP= (F_{ms}-F_s)/(F_{ms}-F_{od})$, and non-photochemical quenching was calculated as $NPQ= (F_m-F_{ms})/(F_{ms})$.

The data obtained from the chlorophyll fluorescence measurements were expressed as means ± standard error. Statistical analyses were performed using the Number Cruncher Statistical System (NCSS) 6.0.7 software package (NCSS, Kaysville, USA). The data were subjected to analysis of variance for repeated measurements, One-way ANOVA, followed by Bonferroni's (All-Pairwise) multiple comparison test. Differences were considered statistically significant at $P < 0.05$.

ACKNOWLEDGMENTS

This work was supported by a seed grant from the Virginia Tech ASPIRES program to BSJW and DRD. The authors thank Kris DeCourcy (Fralin Biotechnology

Center, Virginia Tech) for providing expertise in confocal microscopy, Michael Grusak (USDA-ARS Children's Nutrition Research Center) for performing the analysis of whole plant iron content, and Jonathan Horton (Department of Biology, Virginia Tech) for assistance with chlorophyll fluorescence measurements.

REFERENCES

Abdelnoor, R.V., Yule, R., Elo, A., Christensen, A.C., Meyer-Gauen, G., and Mackenzie, S.A. (2003). Substoichiometric shifting in the plant mitochondrial genome is influenced by a gene homologous to MutS. Proc. Natl. Acad. Sci. U.S.A. **100,** 5968-5973.

Allikmets, R., Raskind, W.H., Hutchinson, A., Schueck, N.D., Dean, M., and Koeller, D.M. (1999). Mutation of a putative mitochondrial iron transporter gene (ABC7) in X-linked sideroblastic anemia and ataxia (XLSA/A). Hum. Mol. Gen. **8,** 743-749.

Amrani, L., Primus, J., Glatigny, A., Arcangeli, L., Scazzocchio, C., and Finnerty, V. (2000). Comparison of the sequences of the *Aspergillus nidulans hxB* and *Drosophila melanogaster ma-l* genes with *nifS* from *Azotobacter vinelandii* suggests a mechanism for the insertion of the terminal sulphur atom in the molybdopterin cofactor. Mol. Microbiol. **38,** 114-125.

Becker, D. (1990). Binary vectors which allow the exchange of plant selectable markers and reporter genes. Nucl. Acids. Res. **18,** 203-210.

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

Bittner, F., Oreb, M., and Mendel, R.R. (2001). ABA3 is a molybdenum cofactor sulfurase required for activation of aldehyde oxidase and xanthine dehydrogenase in *Arabidopsis thaliana.* J. Biol. Chem. **276,** 40381-40384.

Carrington, J.C., and Freed, D.D. (1990). Cap-independent enhancement of translation by a plant potyvirus 5' non-translated region. J. Virol. **64,** 1590-1597.

Chen, H., Nelson, R.S., and Sherwood, J.L. (1994). Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. Biotechniques **16,** 664-668, 670.

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

Csere, P., Lill, R., and Kispal, G. (1998). Identification of a human mitochondrial ABC transporter, the functional orthologue of yeast Atm1p. FEBS Lett. **441,** 266-270.

Flint, D.H. (1996). *Escherichia coli* contains a protein that is homologous in function and N-terminal sequence to the protein encoded by the *nifS* gene of *Azotobacter vinelandii* and that can participate in the synthesis of the Fe-S cluster of dihydroxy-acid dehydratase. J. Biol. Chem. **271,** 16068-16074.

Frazzon, J., and Dean, D.R. (2001). Feedback regulation of iron-sulfur cluster biosynthesis. Proc. Natl. Acad. Sci. U.S.A. **98,** 14751-14753.

Frazzon, J., and Dean, D.R. (2003). Formation of iron-sulfur clusters in bacteria: an emerging field in bioinorganic chemistry. Curr. Op. Chem. Biol. **7,** 166-173.

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

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

Garland, S.A., Hoff, K., Vickery, L.E., and Culotta, V.C. (1999). *Saccharomyces cerevisiae* ISU1 and ISU2: members of a well-conserved gene family for iron-sulfur cluster assembly. J. Mol. Biol. **294,** 897-907.

Gerber, J., and Lill, R. (2002). Biogenesis of iron-sulfur proteins in eukaryotes: components, mechanism and pathology. Mitochondrion **2,** 71-86.

Gornall, A.G., Bardawill, C.J., and David, M.M. (1949). Determination of serum proteins by means of the biuret reaction. J. Biol. Chem.**,** 751-766.

Greb, T., Clarenz, O., Schäfer, E., Müller, D., Herrero, R., Schmitz, G., and Theres, K. (2003). Molecular anallysis of the *LATERAL SUPPRESSOR* gene in *Arabidopsis* reveals a conserved control mechanism for axillary meristem formation. Genes Dev. **17,** 1175-1187.

Gu, J., Miles, D., and Newton, K.J. (1993). Analysis of leaf sectors in the NCS6 mitochondrial mutant of maize. Plant Cell **5,** 963-971.

Hibino, T., Waditee, R., Araki, E., Ishikawa, H., Aoki, K., Tanaka, Y., and Takabe, T. (2002). Functional characterization of choline monooxygenase, an enzyme for betaine synthesis in plants. J. Biol. Chem. **277,** 41352-41360.

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. U.S.A. **97,** 7790-7795.

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

Jaschkowitz, K., and Seidler, A. (2000). Role of a NifS-like protein from the cyanobacterium *Synechocystis* PCC 6803 in the maturation of FeS proteins. Biochem. **39,** 3416-3423.

Kaiser, J.T., Clausen, T., Bourenkow, G.P., Bartunik, H.-D., Steinbacher, S., and Huber, R. (2000). Crystal structure of a NifS-like protein from *Thermotoga*

maritima: implications for iron sulphur cluster assembly. J. Mol. Biol. **297,** 451-464.

Kiley, P.J., and Beinert, H. (2003). The role of Fe-S proteins in sensing and regulation in bacteria. Curr. Opin. Microbiol. **6,** 181-185.

Kispal, G., Csere, P., Prohl, C., and Lill, R. (1999). The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. EMBO J. **18,** 3981-3939.

Köhler, R.H., Cao, J., Zipfel, W.R., Webb, W.W., and Hanson, M.R. (1997). Exchange of protein molecules through connections between higher plant plastids. Science **276,** 2039-2042.

Kolman, C., and Soll, D. (1993). SPL1-1, a *Saccharomyces cerevisiae* mutation affecting tRNA splicing. J. Bacteriol. **175,** 1433-1442.

Kopriva, S., Büchert, T., Fritz, G., Suter, M., Weber, M., Benda, R., Schaller, J., Feller, U., Schürmann, P., Schünemann, V., Trautwein, A.X., Kroneck, P.M.H., 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.

Kubasek, W.L., Shirley, B.W., McKillop, A., Goodman, H.M., Briggs, W., and Ausubel, F.M. (1992). Regulation of flavonoid biosynthetic genes in germinating Arabidopsis seedlings. Plant Cell **4,** 1229-1236.

Kurihara, T., Mihara, H., Kato, S., Yoshimura, T., and Esaki, N. (2003). Assembly of iron-sulfur clusters mediated by cysteine desulfurases, IscS, CsdB and CSD, from *Escherichia coli*. Biochim. Biophys. Acta **1647,** 303-309.

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.

Land, T., and Rouault, T.A. (1998). Targeting of a human iron-sulfur cluster assembly enzyme, nifs, to different subcellular compartments is regulated through alternative AUG utilization. Mol. Cell **2,** 807-815.

Lange, H., Lisowsky, T., Gerber, J., Muhlenhoff, U., Kispal, G., and Lill, R. (2001). An essential function of the mitochondrial sulfhydryl oxidase Erv1p/ALR in the maturation of cytosolic Fe/S proteins. EMBO Rep. **2,** 715-720.

Lauhon, C.T. (2002). Requirement for IscS in biosynthesis of all thionucleosides in *Escherichia coli*. J. Bacteriol. **184,** 6820-6829.

Leibrecht, I., and Kessler, D. (1997). A novel L-cysteine/cystine C-S-lyase directing [2Fe-2S] cluster formation of *Synechocystis* ferredoxin. J. Biol. Chem. **272,** 10442-10447.

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

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

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

Marentes, E., and Grusak, M.A. (1998). Iron transport and storage within the seed coat and embryo of developing seeds of pea (*Pisum sativum* L.). Seed Sci. Res. **8,** 367-375.

Maxwell, K., and Johnson, G.N. (2000). Chlorophyll fluorescence--a practical guide. J. Exp. Bot. **51,** 659-668.

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

Mitsuhashi, N., Miki, T., Senbongi, H., Yokoi, N., Yano, H., Miyazaki, M., Nakajima, N., Iwanaga, T., Yokoyama, Y., Shibata, T., and Seino, S. (2000). MTABC3, a novel mitochondrial ATP-binding cassette protein involved in iron homeostasis. J. Biol. Chem. **275,** 17536-17540.

Muller, K., Matzanke, B.F., Schunemann, V., Trautwein, A.X., and Hantke, K. (1998). FhuF, an iron-regulated protein of *Escherichia coli* with a new type of [2Fe-2S] center. Eur. J. Biochem. **258,** 1001-1008.

Nakai, Y., Nakai, M., Hayashi, H., and Kagamiyama, H. (2001). Nuclear localization of yeast Nfs1p is required for cell survival. J. Biol. Chem. **276,** 8314-8320.

Nakamura, T., Yamaguchi, Y., and Sano, H. (2000). Plant mercaptopyruvate sulfurtransferases. Molecular cloning, subcellular localization and enzymatic activities. Eur. J. Biochem. **267,** 5621-5630.

Nakamura, Y., Kaneko, T., Sato, S., Ikeuchi, M., Katoh, H., Sasamoto, S., Watanabe, A., Iriguchi, M., Kawashima, K., Kimura, T., Kishida, Y., Kiyokawa, C., Kohara, M., Matsumoto, M., Matsuno, A., Nakazaki, N., Shimpo, S., Sugimoto, M., Takeuchi, C., Yamada, M., and Tabata, S. (2002). Complete genome structure of the thermophilic cyanobacterium T*hermosynechococcus elongatus* BP-1. DNA Res. **In press**.

Nilsson, K., Lundgren, H.K., Hagervall, T.G., and Bjork, G.R. (2002). The cysteine desulfurase IscS is required for synthesis of all five thiolated nucleosides present in tRNA from *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **184,** 6830-6835.

Olson, J.W., Agar, J.N., Johnson, M.K., and Maier, R.J. (2000). Characterization of the NifU and NifS Fe-S cluster formation proteins essential for viability in *Helicobacter pylori*. Biochemistry **39,** 16213-16219.

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 E. coli. J. Biol. Chem. **in press**.

Padmasree, K., Padmavathi, L., and Raghavendra, A.S. (2002). Essentiality of mitochondrial oxidative metabolism for photosynthesis: optimization of carbon

assimilation and protection against photoinhibition. Crit. Rev. Biochem. Mol. Biol. **37,** 71-119.

Papenbrock, J., and Schmidt, A. (2000). Characterization of a sulfurtransferase from *Arabidopsis thaliana*. Eur. J. Biochem. **267,** 145-154.

Patzer, S.I., and Hantke, K. (1999). SufS is a NifS-like protein, and SufD is necessary for stability of the [2Fe-2S] FhuF protein in *Escherichia coli*. J. Bacteriol. **181,** 3307-3309.

Pilon, M., America, T., van't Hof, R., de Kruiff, B., and Weisbeek, P. (1995). Protein translocation into chloroplasts. In Advances in Molecular and cell Biology: 1. Membrane Protein Transport, S.S. Rothman, ed (Greenwich, CT: JAI Press), pp. 229-255.

Pilon-Smits, E.A., 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.

Rathinasabapathi, B., Burnet, M., Russell, B.L., Gage, D.G., 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.

Roussell, D.L., Thompson, D.L., Pallardy, S.G., Miles, D., and Newton, K.J. (1991). Chloroplast structure and function is altered in the NCS2 maize mitochondrial mutant. Plant Physiol. **96,** 232-238.

Sakamoto, W., Kondo, H., Murata, M., and Motoyoshi, F. (1996). Altered mitochondrial gene expression in a maternal distorted leaf mutant of Arabidopsis induced by *chloroplast mutator*. Plant Cell **8,** 1377-1390.

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.

Scott, A., Wyatt, S., Tsou, P.L., Robertson, D., and Allen, N.S. (1999). Model system for plant cell biology: GFP imaging in living onion epidermal cells. Biotechniques **6,** 1128-1132.

Sekimoto, H., Seo, M., Kawakami, N., Komano, T., Desloire, S., Liotenberg, S., Marion-Poll, A., Caboche, M., Kamiya, Y., and Koshiba, T. (1998). Molecular cloning and characterization of aldehyde oxidases in *Arabidopsis thaliana*. Plant Cell Physiol. **39,** 433-442.

Seo, M., Peeters, A.J., Koiwai, H., Oritani, T., Marion-Poll, A., Zeevaart, J.A., Koornneef , M., Kamiya, Y., and Koshiba, T. (2000). The Arabidopsis aldehyde oxidase 3 (AAO3) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. Proc. Natl. Acad. Sci. USA **97,** 12908-12913.

Sipos, K., Lange, H., Fekete, Z., Ullmann, P., Lill, R., and Kispal, G. (2002). Maturation of cytosolic iron-sulfur proteins requires glutathione. J. Biol. Chem. **277,** 26944-26949.

Tachezy, J., Sanchez, L.B., and Muller, M. (2001). Mitochondrial type iron-sulfur cluster assembly in the amitochondriate eukaryotes *Trichomonas vaginalis* and

Giardia intestinalis, as indicated by the phylogeny of IscS. Mol. Biol. Evol.**,** 1919-1928.

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

Takahashi, Y., Mitsui, A., and Matsubara, H. (1991). Formation of the Fe-S cluster of ferredoxin in lysed spinach chloroplasts. Plant Physiol. **95,** 97-103.

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

Tong, W.H., and Rouault, T. (2000). Distinct iron-sulfur cluster assembly complexes exist in the cytosol and mitochondria of human cells. EMBO J. **19,** 5692-5700.

Tong, W.H., Jameson, G.N., Huynh, B.H., and Rouault, T.A. (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. U.S.A. **100,** 9762-9767.

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.

von Arnim, A.G., Deng, X.W., and Stacey, M.G. (1998). Cloning vectors for the expression of green fluorescent protein fusion proteins in transgenic plants. Gene **221,** 35-43.

Wollenberg, M., Berndt, C., Bill, E., Schwenn, J.D., and 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.

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

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

Zheng, L., White, R.H., Cash, V.L., and Dean, D.R. (1994). Mechanism for the desulfurization of L–cysteine catalyzed by the *nifS* gene product. Biochemistry **33,** 4714–4720.

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.

CHAPTER 3

Role of the *AtISU* genes in iron-sulfur biogenesis in *Arabidopsis*

Ujwala Warek[1], Ana Paula Guedes Frazzon[2], Dennis R. Dean[3],
and Brenda S.J.Winkel[1]

Departments of Biology[1] and Biochemistry[3], Virginia Tech, Blacksburg, VA. USA and
Graduate Program in Cellular and Molecular Biology (PPGBCM/Cbiot)[2], Federal
University of Rio Grande do Sul, Porto Alegre, RS, Brazil

ABSTRACT

The *Arabidopsis* genome contains three genes AtISU1, AtISU2, and AtISU3 with high homology to proteins that serve as scaffolds for [Fe-S] cluster assembly. In transgenic plants, fusions of GFP with AtISU1 and AtISU3 localized exclusively to mitochondria while a fusion to AtISU2 localized in the chloroplasts. Expression levels observed of AtISU1::GFP and AtISU3::GFP were high and those of AtISU2::GFP very low. The promoters of AtISU1 and AtISU2 were constitutively active while AtISU3 promoter activity was not detected during normal growth and development. A survey of the EST database indicated that only AtISU1 is constitutively expressed. These findings suggest that the *AtISU* genes are differentially regulated at the transcriptional and possibly also the posttranscriptional level. Down regulation of the *AtISU* genes in transgenic plants using antisense and RNAi strategies suggests that these genes play a role in hormone biosynthesis, energy production, and primary metabolism. Furthermore, insertional T-DNA mutants are available for *AtISU2* and *AtISU3* indicating that these genes may have functional redundancy with other scaffold proteins including, AtNFU1-5, and AtISA1-4. These observations, together with evidence that AtISU1 and AtNFS1 can assemble [Fe-S] clusters *in vitro,* strongly suggest that the *AtISU* genes participate in [Fe-S] cluster assembly in plants in different subcellular compartments and under different environmental conditions.

INTRODUCTION

Iron-sulfur [Fe-S] clusters are among the oldest, simplest, and most versatile inorganic cofactors found in nature (Beinert, 2000). Cells have exploited the structural and electronic properties of these clusters for a wide range of important activities including electron transfer, catalysis, substrate activation, and environmental sensing. [Fe-S] proteins play an important role in major biological processes including respiration, photosynthesis, and nitrogen fixation. These proteins acquire their clusters by a posttranslational assembly process that occurs in a controlled manner and involves numerous other protein factors (reviewed in Beinert, 2000; Gerber and Lill, 2002; Frazzon and Dean, 2003; Kiley and Beinert, 2003).

The machinery of [Fe-S] cluster assembly was discovered in the nitrogen-fixing bacterium, *Azotobacter vinelandii* (Zheng et al., 1993; Fu et al., 1994; Zheng et al., 1998; Frazzon and Dean, 2001). Extensive genetic studies identified two operons, *nif* (nitrogen-fixation) and *isc* (iron-sulfur cluster), that are involved in [Fe-S] cluster biogenesis. The *nif* operon contains genes that are specifically responsible for assembly of the [Fe-S] cluster in nitrogenase, an enzyme that converts atmospheric nitrogen to ammonia and is expressed only under diazotrophic conditions. The *isc* operon encodes proteins that assemble [Fe-S] clusters for proteins involved in general cellular activities like respiration and carbon assimilation. The *isc* operon contains at least seven genes, *iscRSUA-hscBA-fdx* (Zheng et al., 1998; Frazzon and Dean, 2001), many of which show high homology to the genes of the *nif* operon. *Escherichia coli* also contains an *isc* operon homologous to that of *A. vinelandii* (Takahashi and Nakamura, 1999). Disruption of the *isc* operon in *E. coli* results in reduced activity of many important [Fe-

S] proteins, including succinate dehydrogenase and aconitase (Tokumoto and Takahashi, 2001). Conversely, overexpression of the entire *isc* cluster leads to an increase in the yield of recombinant [Fe-S] holoproteins (Nakamura et al., 1999).

In addition to the *nif* and *isc* operons, a third system, designated *suf* for its role in sulfur mobilization, was found to play a role in [Fe-S] cluster assembly in bacteria and intracellular parasites. The *suf* operon is induced by oxidative stress and iron deprivation and contains six genes, *sufABCDSE*, including an *iscA* homolog, *sufA*, and an *iscS* homolog, *sufS* (Patzer and Hantke, 1999; Ellis et al., 2001; Takahashi and Tokumoto, 2002).

It is becoming apparent that the nif/isc/suf machinery is highly conserved with homologs present in all organisms examined to date, including bacteria, archaeabacteria, yeast, animals and plants (Lill and Kispal, 2000; Mihara and Esaki, 2002). Specific functions have been assigned to many of the components of this machinery and some progress has been made in understanding the complex network of protein-protein interactions that exist among the [Fe-S] cluster assembly proteins. There appears to be a sequential association and dissociation among the various players that is critical to the assembly of these clusters (Gerber and Lill, 2002; Tokumoto et al., 2002). Among the major players are the NifS/IscS homologs pyridoxal-5'-phosphate (PLP)-dependant enzymes that can catalyze the release of elemental sulfur from L-cysteine to provide the inorganic sulfur necessary for [Fe-S] cluster formation (Zheng et al., 1994; Flint, 1996). These proteins are highly conserved in sequence and function.

The NifU homologs are more diverse in their structures. The NifU protein has a modular structure composed of three domains (Agar et al., 2000c). The N-terminal domain functions as a scaffold during the [Fe-S] cluster assembly process and contains three conserved cysteine residues that constitute a labile mononuclear iron-binding site (Agar et al., 2000b). The bacterial IscU and yeast Isu proteins have high sequence identity to this domain (Strain et al., 1998; Zheng et al., 1998; Garland et al., 1999; Schilke et al., 1999). The central domain of the NifU protein contains a permanent [2Fe-2S] cluster and shows sequence similarity to internal domains of nitrate and nitrite reductases (Ouzounis et al., 1994). The precise function of the C-terminal domain of NifU, which contains a CXXC motif, is not yet known. However, mutants in which these conserved cysteines were substituted with alanine showed a decrease in growth rate under diazotrophic conditions (Agar et al., 2000c). The cyanobacterium, *Synechocystis PCC6803*, yeast, humans, and plants contain proteins designated SyNifU, Nfu1p, Nfu1-2, and AtNFU1-5 respectively, that share homology with the C-terminal domain of NifU (Masepohl et al., 1993; Mühlenhoff and Lill, 2000; Nishio and Nakai, 2000; Léon et al., 2003; Tong et al., 2003).

IscA appears to function as an alternative scaffold for [Fe-S] cluster assembly, perhaps providing for the assembly of other types of clusters, i.e. $[2Fe\text{-}2S]^{2+}$ or $[4Fe\text{-}4S]^{2+}$, targeting different acceptor proteins, or providing activity under different physiological conditions (Krebs et al., 2001). Arabidopsis contains four genes, AtISA1-4, that are 30-40% homologous to *IscA*. Interestingly, iscA homologs have not been identified in any of the Archaeabacterial genomes sequenced to date (Mühlenhoff and Lill, 2000).

The ability of Azotobacter NifU, IscU, and IscA to serve as scaffolds for intermediate cluster assembly has been confirmed by the *in vitro* transfer of clusters to apoferredoxins (Agar et al., 2000c; Agar et al., 2000a; Nishio and Nakai, 2000; Yuvaniyama et al., 2000; Krebs et al., 2001; Ollagnier-de-Choudens et al., 2001; Mansy et al., 2002). It is generally assumed that IscU proteins are the key players in the cluster assembly mechanism as confirmed by the observation that, although both IscU and IscA can form complexes with Fdx, only IscU can make a complex with IscS (Agar et al., 2000b; Kato et al., 2000; Smith et al., 2001; Urbina et al., 2001). Thus it is intriguing that the *suf* operon lacks an IscU homolog but has an IscA homolog (SufA). The SufA protein can act as a scaffold for the formation of $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ clusters that can be transferred to apoproteins (Ollagnier-de-Choudens et al., 2001), suggesting that IscA and SufA do indeed play an important role in transient [Fe-S] cluster assembly and delivery to target proteins. Furthermore, in *E. coli,* which has three cysteine desulfurases designated IscS, SufS (CsdB) and cysteine sufinate desulfurase (CSD), surface plasmon resonance showed that proteins other than IscU act as scaffold proteins for the CsdB and CSD during [Fe-S] cluster formation (Kurihara et al., 2003).

However, some organisms seem to use an assembly mechanism that is either partially or completely different. For example, the cyanobacterium *Synechocystis* PCC 6803 does not contain a complete set of isc components but does contain three IscS-like homologs, all of which have the ability to incorporate [Fe-S] clusters into apo-[Fe-S] proteins (Leibrecht and Kessler, 1997; Jaschkowitz and Seidler, 2000; Kato et al., 2000). In addition, the cyst(e)ine lyase C-DES class of enzymes in cyanobacteria can

also function in [Fe-S] cluster assembly. The existence of other [Fe-S] biogenesis mechanism(s) in prokaryotes is further suggested by the fact that some Archaea, despite containing [Fe-S] proteins, do not possess any homologous components to the isc machinery.

Most of the information on the eukaryotic [Fe-S] clusters assembly mechanism is based on investigations using *Saccharomyces cerevisiae.* Mature [Fe-S] proteins in eukaryotes are found in mitochondria, chloroplasts, the nucleus, and the cytoplasm (Craig et al., 1999; Lill et al., 1999). In yeast, [Fe-S] clusters have been shown to be assembled in the mitochondria and then either inserted into resident mitochondrial proteins or exported to the cytoplasm (reviewed in Gerber and Lill, 2002). The mechanism by which the labile [Fe-S] cluster is exported from mitochondria is not yet fully known, but involves an ABC transporter and appears to require a mitochondrial membrane potential (Kispal et al., 1999; Lange et al., 2001). The [Fe-S] cluster assembly mechanism is apparently compartmentalized in all eukaryotes, with evidence for additional machineries in the cytoplasm in humans and in chloroplasts of plants (Lill and Kispal, 2000; Tong and Rouault, 2000; Léon et al., 2003; Roy et al., 2003).

The main proteins that mediate [Fe-S] cluster assembly in yeast include the functional counterparts of bacterial NifS/IscS (Nfs1p), the NifU C-terminus (NFU1), the NifU N-terminus (Isu1p and Isu2p), IscA (Isa1p and Isa2p), fdx (Yah1p), and two heat shock cognate proteins (Ssq1p and Jac1p), all of which are localized in the mitochondria (Garland et al., 1999; Kaut et al., 2000; Lange et al., 2000; Lill and Kispal, 2000; Pelzer et al., 2000). The current working model for [Fe-S] cluster assembly in yeast is that iron binds to the proteins, Isu1p/Isu2p, and Nfs1p generates the elemental

sulfur to produce an [Fe-S] cluster on a dimer of the Isu proteins (Strain et al., 1998; Garland et al., 1999; Kispal et al., 1999; Lange et al., 2000). Upon reduction, presumably by the mitochondrial ferredoxin Yah1p (Lange et al., 2000), the cluster is released to the apoproteins. Further, yeast frataxin (Yfh1) is suggested to play a role in loading iron on the Isu scaffold proteins (Gerber et al., 2003). The chaperones Ssq1 and Jac1 interact with Isu1/2 and are required after [Fe-S] assembly (Dutkiewicz et al., 2003; Mühlenhoff et al., 2003). Deletion of either *ISU1* or *ISU2* genes in yeast results in increased accumulation of iron within mitochondria, loss of the $[4Fe\text{-}4S]^{2+}$ aconitase activity, and suppression of oxidative damage in cells lacking cytosolic copper/zinc superoxide dismutase. These genes appear to be regulated by the iron status of the cell and Isu1p is the predominantly expressed form under most conditions (Garland et al., 1999).

In contrast to bacteria and yeast, very little is known about the [Fe-S] cluster assembly mechanism in plants. Evidence indicates the presence of distinct machineries in chloroplasts and mitochondria. This suggests that plants may have two different metabolic systems, each of which targets a distinct array of cellular [Fe-S] proteins. There is good evidence that mitochondria in plants are able to synthesize, assemble, and export [Fe-S] clusters. *In vitro* studies have shown that plant mitochondrial extracts can reconstitute the [Fe-S] protein maturation process of biotin synthase in the presence of reduced iron, ATP and NADH (Mühlenhoff et al., 2002). Chloroplasts are competent in [Fe-S] cluster assembly in the absence of mitochondria (Merchant and Dreyfuss, 1998). Furthermore, lysed spinach chloroplasts were shown to support the formation of endogenous [Fe-S] clusters targeted for ferredoxin maturation

in vitro, indicating the existence of [Fe-S] cluster biogenesis machinery in plastids (Takahashi et al., 1991), a finding that was confirmed and extended by Pilon et al. (1995).

Some efforts have been made to identify the components of these machineries. An earlier hypothesis proposed that plant mercaptopyruvate sulfutransferases (MST) homologs acted as the sulfur donors by transferring sulfur ions from mercaptopyruvate to cyanide ions. Of the two *Arabidopsis* MST's present, one is located in mitochondria and the other in the cytoplasm. A T-DNA knockout mutant of the mitochondrial MST1, however, had no apparent effect on [Fe-S] enzyme activities, therefore suggesting that MST1 is not directly involved in iron-sulfur cluster assembly (Nakamura et al., 2000; Papenbrock and Schmidt, 2000).

Arabidopsis contains a single gene, *AtNFS1,* that has high amino acid identity with *A. vinelandii* IscS (Kushnir et al., 2001; Tachezy et al., 2001; Léon et al., 2002a). Recent work has shown that AtNFS1 has cysteine desulfurase activity, is localized exclusively in mitochondria, and that transgenic plants expressing *AtNFS1* in the antisense orientation exhibit phenotypes consistent with a role for this gene in hormone biosynthesis, the response to oxidative stress, and sulfur assimilation (Chapter 2; Ana Frazzon, unpublished). Two independent studies characterized an *Arabidopsis* protein designated AtNFS2 (or ATCpNIFS), that is most closely related to SufS. This protein is localized to chloroplasts as determined experimentally using an *in vitro* chloroplast import assay and by localization of GFP fusion proteins in protoplasts (Léon et al., 2002a; Pilon-Smits et al., 2002). Like *E. coli* SufS, AtNFS2 has a much higher

desulfurase activity with selenocysteine than cysteine, suggesting a function distinct from or in addition to [Fe-S] cluster assembly (Pilon-Smits et al., 2002).

Arabidopsis also contains five genes (*AtNFU1-5*) with homology to the C-terminus of NifU that can complement a yeast *Δisu1Δnfu1* mutant, confirming their role in [Fe-S] cluster biogenesis (Léon et al., 2003). These proteins share the conserved CXXC motif in their NFU domain and can be separated into two classes depending on the number and location of this motif. AtNFU1-3 localize to chloroplasts and represent a new class of proteins that have two NFU domains and are most closely related to *Synechocystis* PCC6803 SyNifU (Mühlenhoff et al., 2002; Léon et al., 2003). AtNFU4 and AtNFU5 localize to mitochondria and have only one NFU-like domain whose location in the polypeptide is different than in ATNFU1-3, and presents a structural organization that is similar to *S. cerevisiae* Nfu1p (Léon et al., 2003). Kushnir et al. (2001) identified three genes (*AtSTA1-3*) in *Arabidopsis* with homology to the yeast ABC transporter *ATM1p* which has been shown to perform a crucial function in the maturation of cytoplasmic [Fe-S] proteins. AtSTA1 could complement the function of the Atm1p transporter leading to almost normal biosynthesis of cytoplasmic proteins (Csere et al., 1998; Bekri et al., 2000; Kushnir et al., 2001).

Arabidopsis also contains three genes, *AtISU1*, *AtISU2,* and *AtISU3,* which have high sequence similarity with IscU. As part of an effort to further understand the complex system of [Fe-S] cluster assembly in plants, AtISU1-3 were characterized. A search of the EST database indicates that only AtISU1 is expressed at an appreciable level. Insertional mutants are present for AtNFS1, AtISU2, and AtISU3 in the SIGnAL (Salk Institute Genomic Analysis Laboratory, USA) T-DNA collection (Alonso et al.,

2003). This suggests that the proteins encoded by these genes are not essential for survival and other proteins in the plant that have overlapping functions can substitute them.

Analyses of GFP fusions of these proteins in transgenic plants suggested the existence of two distinct [Fe-S] cluster assembly sites, AtISU1 and AtISU3 in the mitochondria, and AtISU2 in the chloroplasts. Promoter analysis indicated that AtISU1 and AtISU2 are constitutively transcribed, whereas AtISU3 transcription was not detected under the conditions examined suggesting that the *AtISU* genes are differentially regulated at the transcriptional and possibly also the post-transcriptional level. Evidence also suggests that AtISU2 mRNA may be unstable. For all three genes, plants in which expression was down-regulated using antisense or RNAi technology, unusual phenotypes that included perturbations in growth rate, patterns of leaf and shoot development, time of bolting, or location of flowers were observed. These observations, together with previous *in vitro* evidence that AtISU1 can form transient [Fe-S] clusters in Arabidopsis NFS1 (Ana Frazzon, unpublished), suggest that the AtISU genes are indeed involved in [Fe-S] cluster assembly in different compartments.

RESULTS

Arabidopsis contains homologs of all of the proteins that have been identified as players in the process of biological [Fe-S] cluster formation in microorganisms and animals (reviewed in Chapter 1). The proteins identified as key players in the "housekeeping" [Fe-S] cluster biogenesis machinery in bacteria are IscS, which acts as

a sulfur donor, and the scaffold-forming protein IscU (Agar et al., 2000b). While chapter 2 discusses in detail the characterization of the *Arabidopsis* IscS homolog, AtNFS1, an initial study of the *Arabidopsis* IscU homologs has also been performed as described below.

Characterization of the *AtISU* genes

Arabidopsis contains three genes, *AtISU1* (At4g22220), *AtISU2* (At4g04080), and *AtISU3* (At3g01020), that have high sequence similarity (65.6-67.2 % amino acid identity) with *Azotobacter* IscU (Figure 1). The *AtISU1-3* genes each contain two introns at identical locations and are more closely related to each other (62.5-65.9 % overall amino acid identity and 66.1-76.1 % amino acid identity without the putative transit peptides) than to proteins from other species (Figure 2). However, these genes are not linked in the *Arabidopsis* genome (Table 1) indicating that the generation of redundant copies was not a recent event. A survey of the EST database indicates that *AtISU1* is expressed in a variety of tissues, including seeds, flowers, roots, and leaves, while *AtISU2* and *AtISU3* are not expressed at a significant level in any tissues (Table 1).

Subcellular Localization of the AtISU proteins

AtISU1, AtISU2, and AtISU3 contain N-terminal sequences that extend beyond the region of homology with *Azotobacter* IscU (Figure 1). PSORT and TargetP analysis indicate that these sequences could serve as signal peptides for translocation of AtISU1 to mitochondria and of AtISU2 and AtISU3 to either mitochondria and/or chloroplasts.

Figure 1. Sequence alignment of *Arabidopsis AtISU* genes and *Azotobacter IscU*.

Amino acid sequence alignment of the AtISU1 (accession number 15235569), AtISU2 (15233385), AtISU3 (15232028), and Azotobacter IscU (AAC24473) proteins. The sequences were aligned by the CLUSTAL W program (Thompson et al., 1994). Identical residues are shaded.

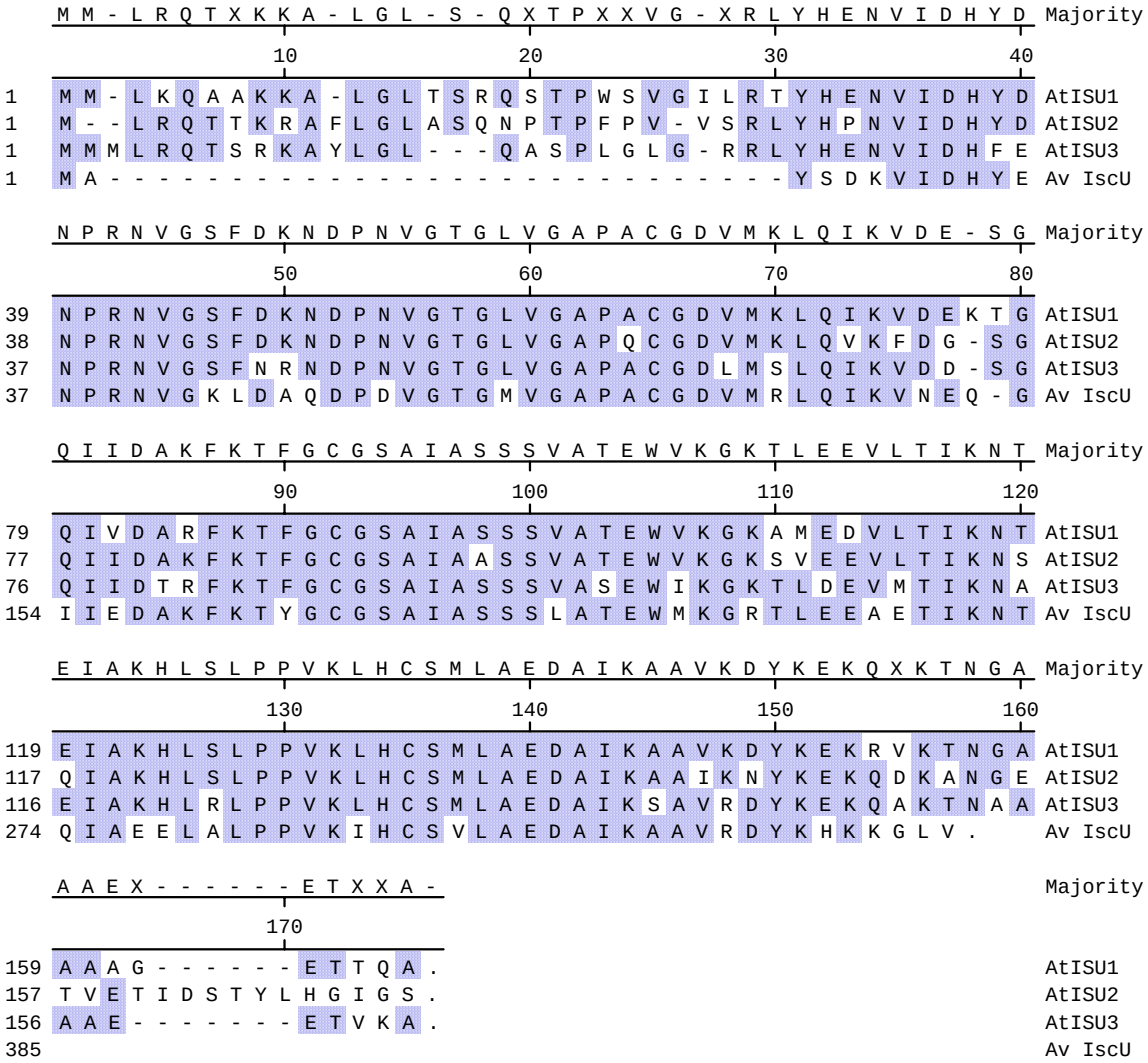


Figure 2. **Phylogenetic relationships among NifU and NifU-like protein sequences from different organisms.** Only sequences with homology to the amino terminus of NifU are included in the analysis. The sequences fall into four defined clusters that reflect the source organism; yeast (pink), multicellular eukaryotes (purple), eubacteria (green), or archaebacteria (gray). These are clearly distinct from the amino terminus sequence of *Azotobacter* NifU involved in the maturation of nitrogenase. The arrows identify the positions of the *Arabidopsis* proteins in this phylogeny.

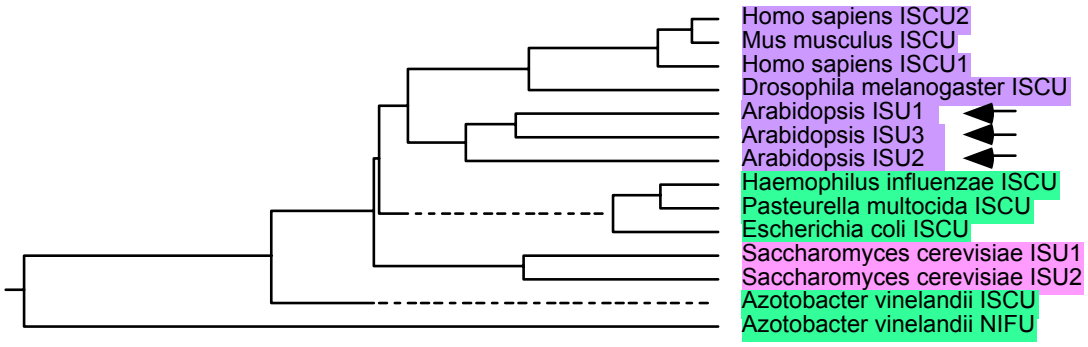


Table 1. Genomic information for the *Arabidopsis* IscU homologs.

Gene	Locus	Chromosome location	EST matches
AtISU1	At4g22220	16,600 kb; chromosome 4	8 (seedlings, leaves, roots, flowers, dehydration- and cold-treated plants at various stages)*
AtISU2	At4g04080	3,400 kb; chromosome 4	0 in database, 1 cDNA from flowers (gift of Stephane Lobreaux Universitè Montpellier, France)
AtISU3	At3g01020	670 kb; chromosome 3	0 in database, 1 cDNA from flowers (gift of Stephane Lobreaux, Universitè Montpellier, France)

*from individual libraries, i.e. not pooled

In order to determine the subcellular location of AtISU1, AtISU2, and AtISU3 proteins, transgenic plants were generated that produced these proteins fused to green fluorescent protein, GFP (Haseloff et al., 1997). The AtISU1::mGFP construct was generated (Ana Frazzon, unpublished) by using the BAC clone K3F7TP as a template to amplify the 165aa *AtISU1* coding region. The resulting fragment was cloned in-frame with the amino terminus of mGFP5 in pAVA393 (von Arnim et al., 1998). The AtISU1::GFP fragment , including the double-enhanced cauliflower mosaic virus CaMV 35S promoter, Tobacco Etch Virus (TEV) translational leader, and nopaline synthase (nos) terminator sequences, were sub cloned in the binary vector, pBIB (Becker, 1990a).

In the case of AtISU2 and AtISU3, due to the unavailability of full-length cDNA's, the coding sequences plus introns but without the stop codon were amplified from genomic DNA. These fragments, 516 bp for *AtISU2* and 492 bp for *AtISU3*, were fused in-frame to the N-terminus of the sequence encoding mGFP5 in the plant binary vector pCAMBIA1302 (Cambia, Canberra, Australia) under control of the double-enhanced CaMV 35S promoter.

Expression constructs were confirmed for the presence of the insert by PCR, restriction digestion, and sequencing. Transgenic *Arabidopsis* ecotype Columbia plants were generated using the floral dip method (Clough and Bent, 1998). Resistance to hygromycin was used to identify transgenic plants. Seedlings expressing detectable levels of GFP were identified based on the intensity of fluorescence in the roots when observed under UV light with a dissecting microscope. Seedlings expressing GFP at a range of levels were then transferred to soil and grown for analysis using confocal laser

scanning microscopy (CLSM). Wild type plants grown under the same conditions were used as controls and autofluorescence in these plants was used as a threshold to account for any background inflorescence due to chlorophyll during CLSM analysis. As a control, plants transformed with the unmodified pCambia1302 vector were used to account for GFP localization in the absence of an ISU fusion partner. Localization was analyzed in leaves of 3-4 week old plants. For determining AtISU1 localization, plants from multiple independent lines of the T1, T2 and T3 generations and one independent T4 generation (high expresser) were examined. Control plants showed GFP accumulation in the cytoplasm but not in mitochondria, chloroplasts, or nuclei. In contrast, AtISU1::GFP was targeted specifically to mitochondria (Figure 3), consistent with predictions from PSORT and TargetP analysis. For AtISU2 and AtISU3 localization, plants from multiple independent lines of T1, T2, T3, and T4 generation were examined. AtISU3::GFP localized specifically to mitochondria (Figure 4). AtISU2::GFP was found localized at relatively low levels only in the chloroplasts in thirteen independent T1 lines (Figure 5). No plants with high-level florescence were detected among over thirty different AtISU2::GFP transgenic lines that were screened. To confirm that the GFP signal was not simply being quenched due to chlorophyll autofluorescence in these plants, total protein was extracted and subjected to immunoblot analysis using an anti-GFP antibody. No detectable GFP signal was observed in any of the protein extracts from the low-level expressers while a positive control consisting of proteins extracts from plants expressing high levels of an endoplasmic-reticulum (ER)-targeted mGFP (Haseloff et al., 1997) gave a strong signal (Figure 6). This indicates that the low level of fluorescence in the AtISU2::GFP was

Figure 3. Subcellular localization of AtISU1::GFP fusion protein in *Arabidopsis* leaves. AtISU1 was fused to the N-terminus of the plant-optimized reporter gene, *mGFP5*, and expressed in transgenic *Arabidopsis* plants under the control of the constitutive double-enhanced CaMV 35S promoter. Leaves from 4 week-old-plants examined by CLSM are shown. GFP fluorescence is shown in green and autofluorescence from chloroplasts is in red. **(A)** Stomata **(B)** closeup of stomata, **(C)** closeup of chloroplast and two mitochondria within the stomata.

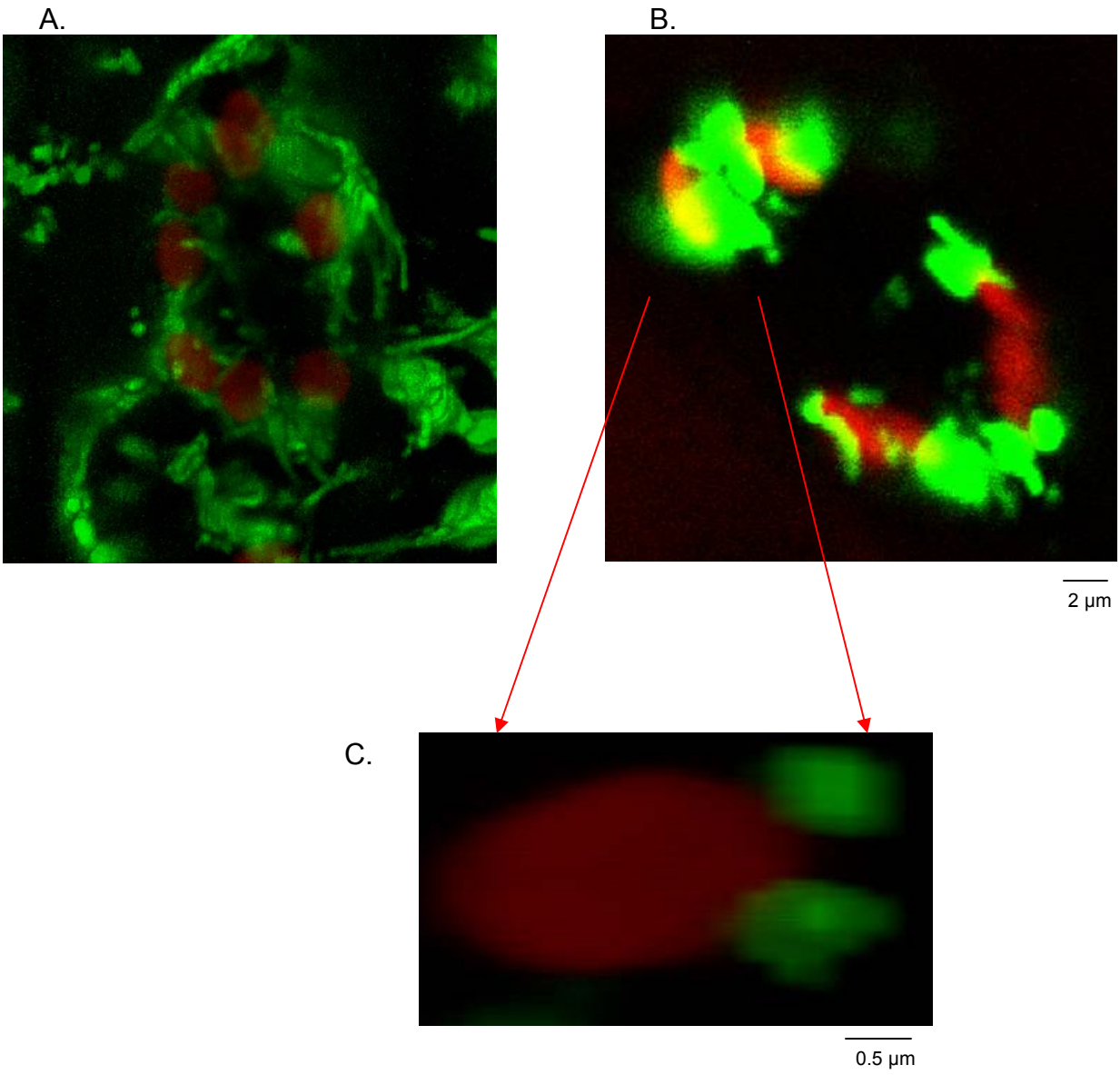
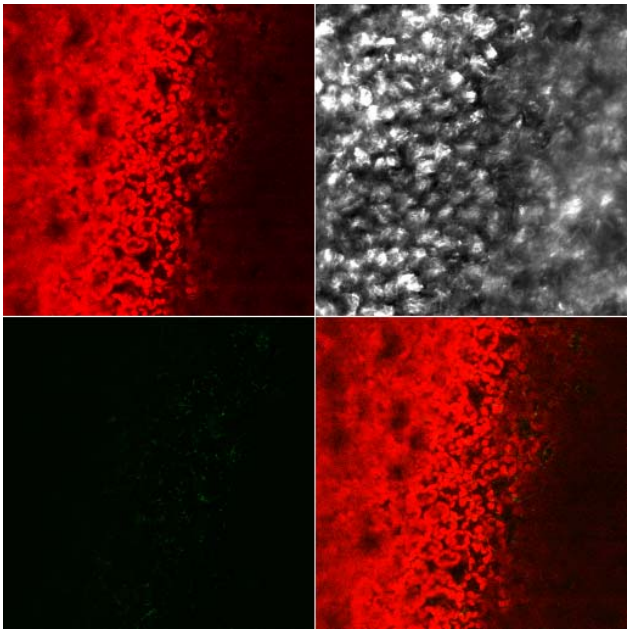


Figure 4. Subcellular localization of AtISU3::GFP fusion protein in *Arabidopsis* leaves. The experiment was performed as described in the legend of Figure 3. **(A)** View showing chloroplast autofluorescence on the upper left, DIC image on upper right, GFP fluorescence on lower left and the merged image on the lower right, **(B)** closeup of merged image of the stomata.

A.



B.

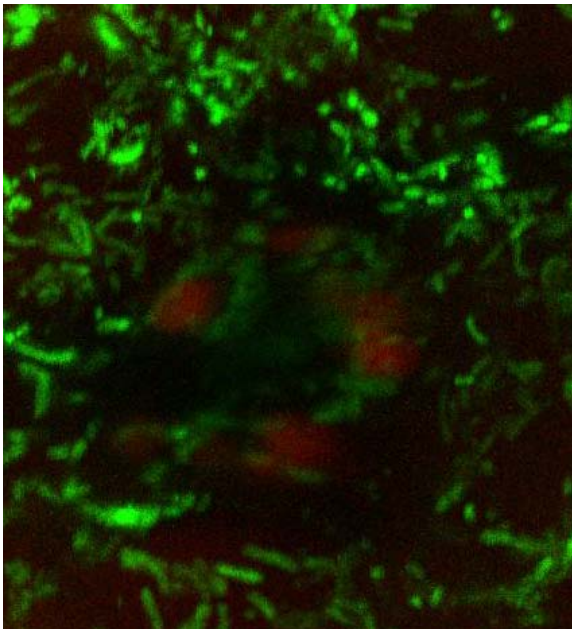


Figure 5. Subcellular localization of AtISU2::GFP fusion protein in *Arabidopsis* and autoflorescence in wildtype plants. The experiment was performed as described in the legend of Figure 3. **(A)** AtISU2::GFP expression. View showing chloroplast autofluorescence on the upper left, DIC image on upper right, GFP fluorescence on lower left and the merged image on the lower right, **(B)** wildtype leaves observed under same conditions.

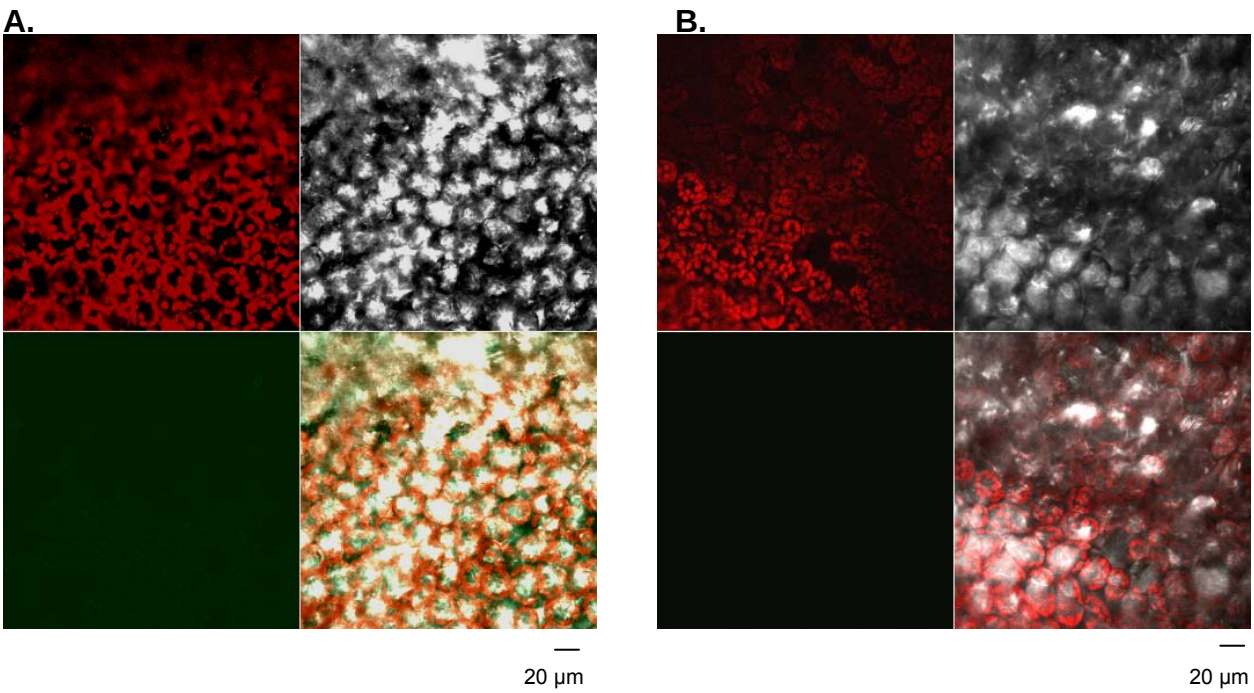
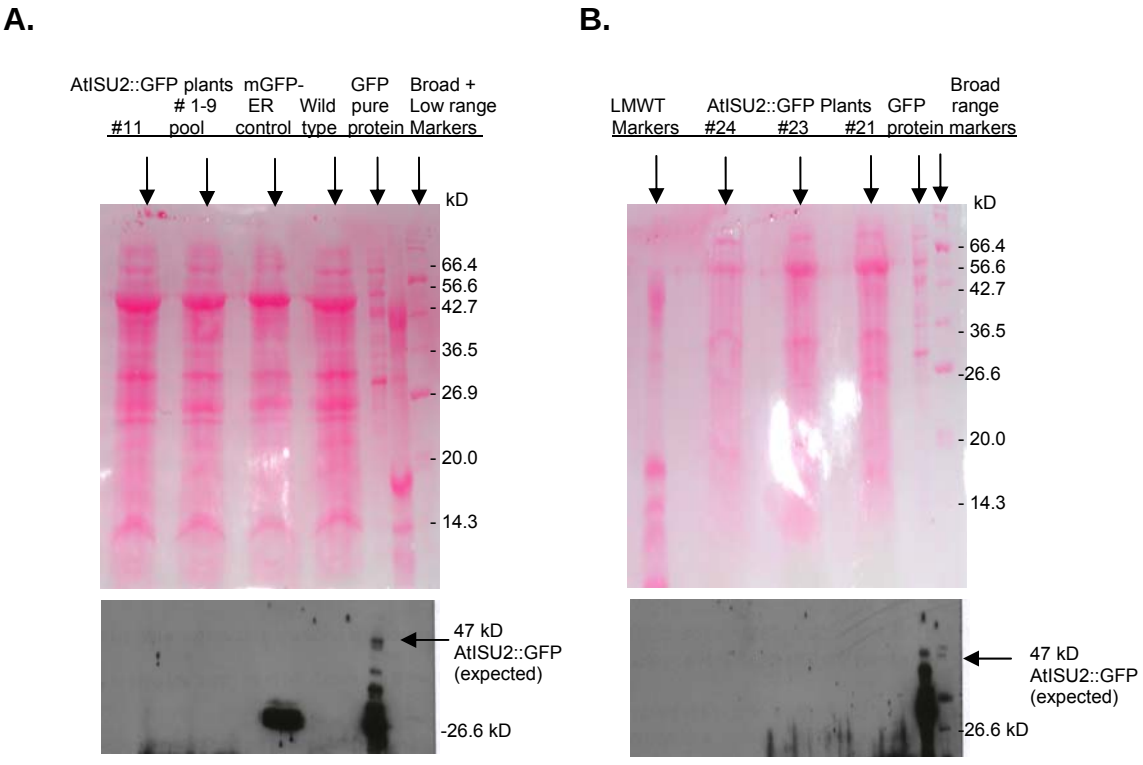


Figure 6. Analysis of GFP levels in AtISU2::GFP plants. Crude protein extracts (75 µg) from 13 different AtISU2::GFP lines, mGFP5-ER plants, and wild-type *Arabidopsis* plants, and purified recombinant GFP protein (1 µg) were fractionated by SDS-PAGE and then analyzed on an immunoblot using anti-GFP antibody. Numbers below indicate the different AtISU2::GFP plant lines. Protein from plant lines 1 through 9 was pooled. **(A)** PonceauS-stained membrane of proteins extracts that were fractionated by SDS PAGE and transferred to the membrane. **(B)** Western Blot using anti-GFP antibody.



due to very low accumulation of the protein product, below detection of the immunoblot assay.

Characterization of AtISU1, AtISU2, and AtISU3 promoter activity

In order to determine the tissue-specific and developmental expression of the *AtISU1, 2* and *3* genes, 5' upstream regions, including the 5' UTR, were fused in-frame with the start codon of the β-glucuronidase (GUS) reporter gene in the plant binary vector, pBW18 (gift of Bonnie Woffenden, McDowell Lab, Virginia Tech). Sequences up to approximately 1 kb upstream of the AtISU genes were considered likely to contain the full regulatory regions unless defined by the presence of an upstream annotated gene. The next upstream annotated gene was at 1410 bp for AtISU1, at 13777 bp for AtISU2, and at 340 bp for AtISU3. Accordingly, regions of 1400 bp, 968 bp, and 340 bp for the *AtISU1*, *AtISU2,* and *AtISU3* genes, respectively, were used in this analysis. Recombinant plasmids were identified by PCR, restriction digestion, and sequencing. Transgenic *Arabidopsis* ecotype Columbia plants were identified based on Basta® resistance and further confirmed for the presence of the transgene by PCR of the plant tissue using primers specific for the insert as well as primers specific for the GUS gene. Multiple independent transformants were identified for each of the constructs, of which ten were selected for further characterization based on the intensity of GUS staining.

To obtain a representative pattern of expression, it was necessary to analyze plants expressing GUS over a range of levels to rule out the possibility that staining patterns were due to the positional effects of neighboring sequences on the transgene. Analysis of the promoter::GUS lines indicated that both *AtISU1* and *AtISU2* promoters

were active in all tissues at 10, 17, and 31 days which represent seedling, bolting and flowering stages of development respectively, suggesting constitutive expression. Figure 7 shows the results for several representative lines at the T2 generation. All T3 generation homozygotes containing the AtISU1 and AtISU2 promoter constructs showed apparent silencing of GUS expression while earlier generations of these lines had exhibited strong GUS expression. On the other hand, for the *AtISU3* promoter, no GUS activity was detected in any of the more than 30 lines that were examined.

Phenotypic effects of altered *AtISU1* expression in transgenic plants

In an effort to gain insight into the physiological function of AtISU1, transgenic plants were generated in which a full length *AtISU1* cDNA was placed in either the forward or reverse orientation behind the double-enhanced 35S promoter (Ana Frazzon, unpublished). It was expected that the resulting transgenic plants would exhibit over-expression or co-suppression of the *AtISU1* gene if the plants contained the gene in the sense orientation and gradations of silencing for the anti-sense construct. For each construct, five independent T1 plants were identified based on resistance to kanamycin, and four homozygous lines were identified in the T4 generation. Three of five independent lines carrying the *AtISU1* sense construct appeared to be larger than normal. Anti-sense expression of *AtISU1* resulted in loss of apical dominance, a bushy phenotype, and appearance of inflorescences in bunches and at the base of the stem as the plants aged (Figure 8). However, phenotypes observed here may not be specific to AtISU1 silencing due to the high sequence similarity of the *AtISU* genes resulting in silencing of all of them. No clear evidence of cosuppression was seen for these genes

Figure 7. Expression patterns of the AtISU1, AtISU2, and AtISU3 promoters. The 5' regulatory regions of the genes were fused in frame to the N-terminus of the GUS gene in pBW18 and expressed in transgenic *Arabidopsis* plants. Top panel: AtISU1, Middle: AtISU2, and Bottom: AtISU3 promoter. **(A)** 10-day-old plants, **(B)** 17-day-old plants, and **(C)** 31-day-old plants were stained for GUS activity; **(D)** closeup of the inflorescence of 31-day-old AtISU2p::GUS plant.

Figure 7D. Closeup of GUS staining in the inflorescence of a 31-day-old AtISU2::GUS plant.

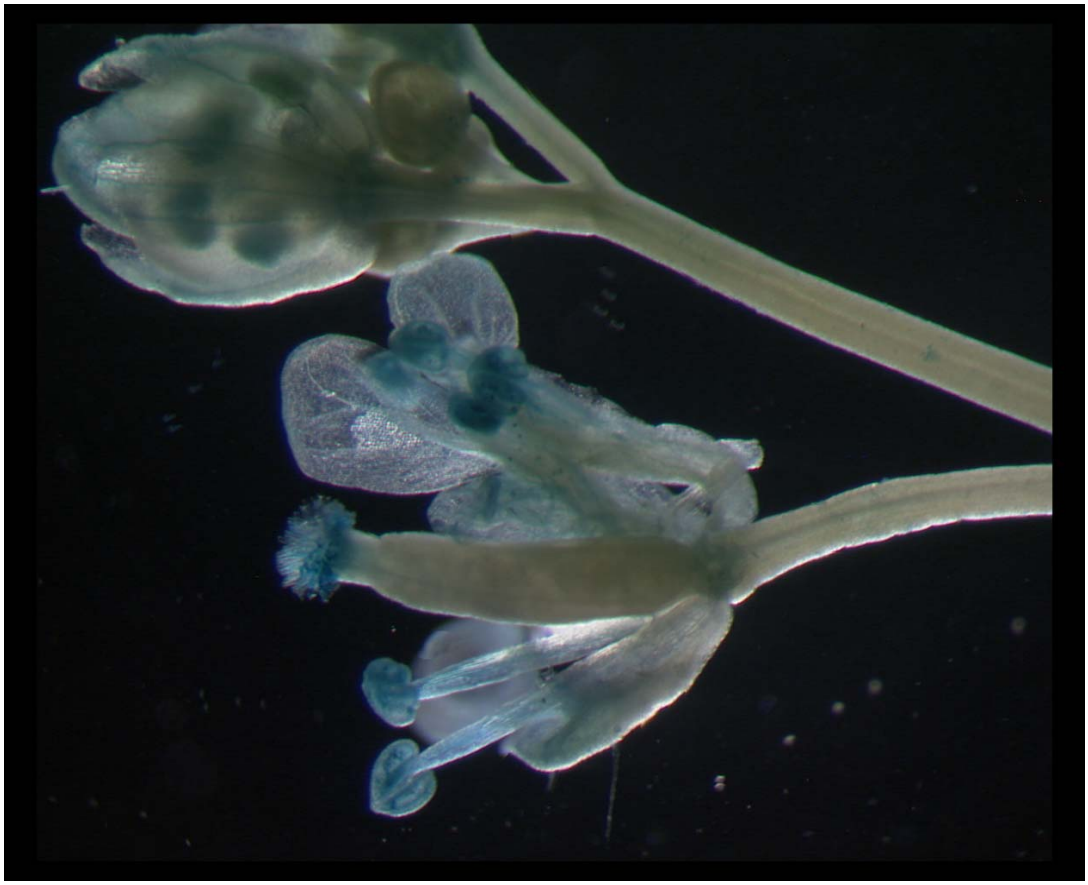
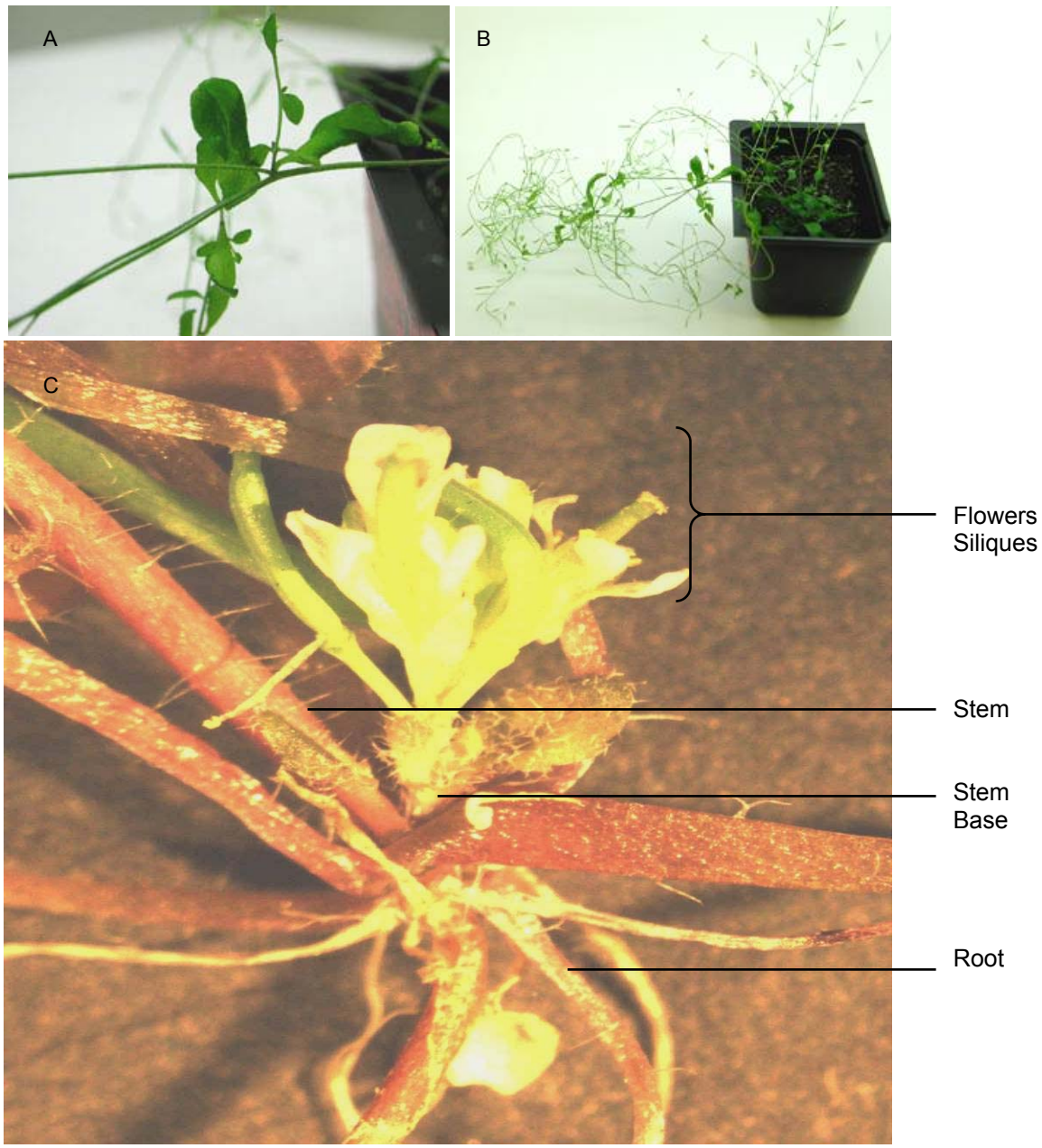


Figure 8. Phenotypes of plants expressing *AtlSU1* in the antisense direction.

(A) Loss of apical dominance, **(B)** bushy phenotype, **(C)** appearance of inflorescence at base of stem base in older plants.



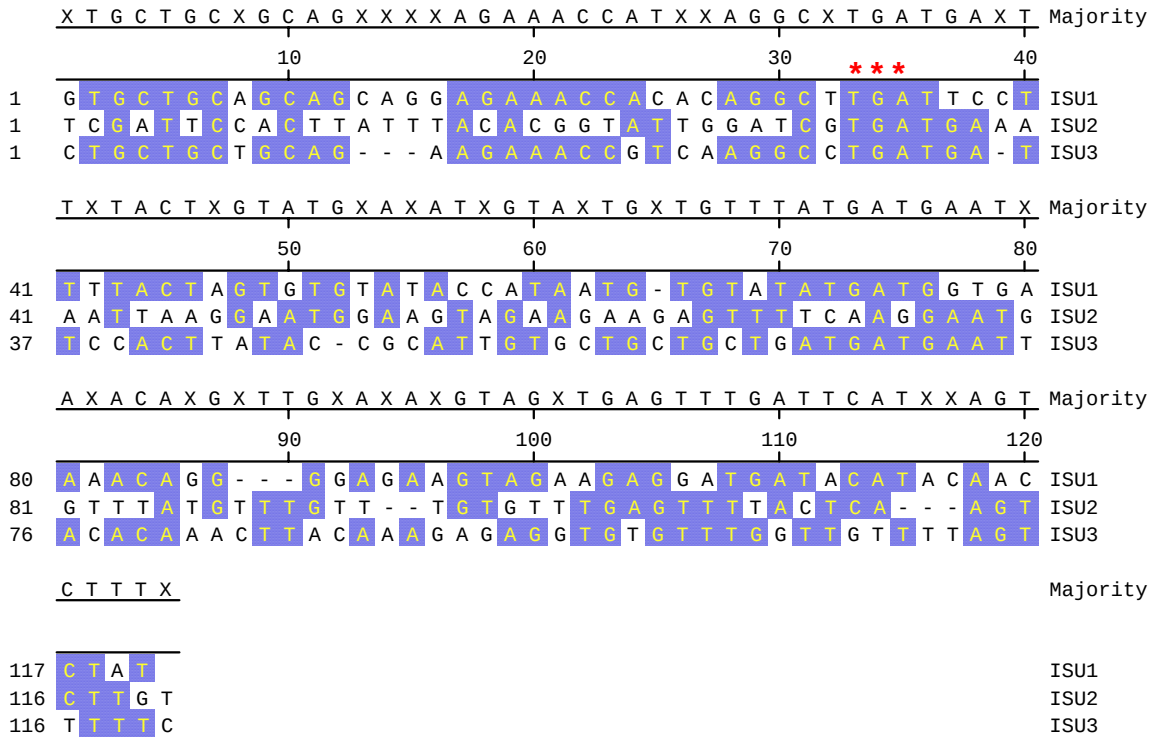
as none of the lines containing the *AtISU1* sense constructs had any of these phenotypes.

Phenotypic effects of silenced *AtISU* genes in transgenic plants

Development of RNAi technology offers opportunities for more effective gene silencing in a more specific manner compared to antisense approaches. Using hairpin RNA (hpRNA) constructs containing unique complementary sense/antisense segments ranging from 98-853 nt from a specific gene and introns, 90-100 % silencing in independent transgenic plants has been achieved (Wesley et al., 2001). In order to begin defining the precise physiological functions of each of the *AtISU* genes, RNAi constructs were designed using 120 bp fragments starting near the 3' end of the coding region and extending into the 3' untranslated (UTR) of each gene. These 120 bp regions, which showed between 24 and 33 % identity at the nucleotide level (Figure 9), were cloned into the pHANNIBAL vector (Wesley et al., 2001). Recombinant plasmids were identified by PCR, restriction digestion, and sequencing. The RNAi cassette was then transferred to the plant binary vector, pCAMBIA3300 (Cambia, Canberra, Australia), and recombinant plasmids were once again identified by PCR, restriction digestion, and sequencing. Transgenic *Arabidopsis* ecotype Columbia plants were identified based on resistance to the herbicide Basta®. Ten independent T1 lines were recovered and analyzed for phenotypic differences for each of the *AtISU2, ATISU3*, and the empty vector constructs. *AtISU1* RNAi silenced plants are still in the process of being identified.

Figure 9. Sequences used in generating RNAi constructs for the AtISU genes.

Nucleotide alignment of the 120bp regions of the *AtISU1*, *AtISU2*, and *AtISU3* genes used for RNAi constructs. Regions for each gene included 32-35 bp at the 3' end of the coding region, extending 85-88 bp into the 3' UTR. The sequences were aligned by the CLUSTAL W program (Thompson et al., 1994). Identical residues are shaded. Stop codons are indicated by red asterisks.



Transgenic plants containing the *AtISU2* RNAi construct showed two distinct sets of phenotypes (Figure 10). One group of plants exhibited accelerated growth rate, disrupted rosette formation, and altered leaf shape relative to wild type. These plants also produced large cauline leaves at the base of the flower. The other group of plants had a slow growth rate but very early onset of bolting and an altered rosette pattern relative to wild type. These plants had thinner stems and were in general smaller in size. Transgenic T1 plants containing the AtISU3 RNAi also had thinner stems and were generally smaller. These plants also exhibited an altered rosette pattern with the appearance of extra rosette-like leaves on the stems (Figure 11). The empty vector control showed no unusual phenotype. In comparison to the *AtISU1* antisense plants, the phenotypes observed for the *AtISU2* and *AtISU3* RNAi plants were more severe. While all showed some form of aberrant inflorescence development, the differences in growth rates, rosette patterns, and stems were not evident in the *AtISU1* silenced plants. This could reflect a difference in the degree of silencing achieved using the two approaches, or could indicate a true difference in the complement of apoproteins targeted by each AtISU isoform.

DISCUSSION

Considerable progress has been made in understanding the biogenesis of [Fe-S] cluster assembly in bacteria, yeast and, to some extent, animals, but very little is known in plants. Genes in plants that have been implicated to play a role in [Fe-S] cluster assembly mechanism include *AtNFS2* (or *ATCpNIFS*), *AtNFU1-5, AtNFS1, AtISA1-4*

Figure 10. Phenotype of 56 day old plants expressing *AtISU2* RNAi.

(A) Wild-type plants and *AtISU2* RNAi plants showing two different phenotypes, accelerated growth-rate (middle) and smaller, weaker plants that showed early onset of bolting. **(B)** Wild-type rosette pattern. **(C)** and **(D)** Altered rosettes pattern of *AtISU2* RNAi plants **(E)** and early onset of bolting in plants expressing *AtISU2* RNAi.

Figure 11. Phenotypes of transgenic plants expressing *AtISU3* RNAi.

(A) Wild-type plants and **(B), (C),** and **(D)** T1 generation *AtISU3* RNAi plants showing growth retardation, disruption in rosette formation and appearance of rosette-like leaves on the stem.

and the mitochondrial ABC transporters *AtSTA1-3* (Kushnir et al., 2001; Pilon-Smits et al., 2002; Léon et al., 2003). Evidence suggests that AtNFS1, AtNFU4, AtNFU5, and AtSTA1 are part of a mitochondrial assembly system analogous to the bacterial isc and yeast machinery, while AtNFS2 and AtNFU1-3 are part of a system located in chloroplasts with greatest similarity to the *E. coli* suf system (Kushnir et al., 2001; Pilon-Smits et al., 2002; Léon et al., 2003).

The present study provides some of the first insights into the role of AtISU1-3, which show high homology (up to 67%) to the NifU/IscU scaffold proteins of the [Fe-S] cluster assembly machinery. The reason for the redundancy of the *AtISU* genes in *Arabidopsis* is not known. Recombinant AtISU1 was previously shown to form transient [Fe-S] clusters with *Arabidopsis* AtNFS1, which supports its role as a scaffold protein (Chapter 2; Ana Frazzon, unpublished). AtISU2 and AtISU3 have not yet been tested for *in vitro* activity. Insertional mutants are available for AtISU2 and AtISU3 in the SIGnAL (Salk Institute Genomic Analysis Laboratory, USA) T-DNA collection (Alonso et al., 2003). This suggests that the AtISU2 and AtISU3 may have overlapping functions with AtISU1, AtNFU1-5, or AtISA1-4. From an evolutionary standpoint, plants have developed a sophisticated network of proteins to compensate for a sessile lifestyle and survive in an ever changing and challenging environment. Thus, it is possible that the redundancy of these genes facilitates the assembly of [Fe-S] clusters under different developmental or environmental conditions in different subcellular compartments and tissues.

Eukaryotic organisms show subcellular compartmentalization of the [Fe-S] cluster assembly mechanism (Mühlenhoff and Lill, 2000; Léon et al., 2003; Tong et al.,

2003). CSLM analysis of GFP fusions in transgenic plants showed that AtISU1 and AtISU3 localized to mitochondria while AtISU2 localized to chloroplasts. This observation was consistent with N-terminal sequence analysis of AtISU1. The differential localization of the AtISU genes suggests the existence of two distinct [Fe-S] cluster assembly sites, one in mitochondria, and the other in the chloroplasts, consistent with evidence that cysteine desulfurases, AtNFS1 (Chapter 1, Kushnir et al., 2001) or AtNFS2, are also located in each of these organelles (Léon et al., 2002b; Pilon-Smits et al., 2002).

An unexpected finding was that, while expression of the GFP fusions of AtISU1 and AtISU3 were high and easily detectable, only low-level accumulation of AtISU2::GFP was observed. Further, immunoblot analysis using an anti-GFP antibody failed to confirm the presence of the AtISU2::GFP fusion protein in any of thirteen transgenic lines tested. Low level accumulation of AtISU2::GFP could be explained by a lack of stability of the AtISU2::GFP mRNA or protein.

It was further speculated that the AtISU genes are differentially expressed because a survey of the EST database indicates that AtISU1 is constitutively expressed while AtISU2 and AtISU3 are not expressed. AtISU2 and AtISU3 cDNA was isolated by S. Lobréaux's group (Université Montpellier, France) from flowers (personal communication). However, efforts in our lab to generate these cDNA's by RT-PCR from a variety of tissues were unsuccessful (unpublished findings). Consistent with these findings, analysis of an AtISU1p::GUS construct in transgenic plants indicated that the AtISU1 promoter is constitutively active, while the AtISU3p::GUS construct was not expressed in plants at a detectable level under any of the conditions tested.

Surprisingly, the AtISU2 regulatory region was found to be active in all tissues and at all developmental stages examined. One explanation for this observation is that the AtISU2 promoter is active but the resulting transcript is highly unstable. This may also explain the inability to isolate lines expressing high levels of AtISU2::GFP plants. mRNA analysis of transgenic plants expressing AtISU2::GFP fusions made with varying lengths of the 5' sequence of AtISU2 may shed some light to these findings. These analyses show that the *AtISU* genes are differentially regulated at the transcriptional and possibly also at the post-transcriptional level. Further studies on the effects of biotic and abiotic stress on AtISU1-3 promoter activity could give some indication on whether there are further differences in the expression of these genes under different environmental conditions. Constitutive expression of AtISU1 and AtISU2 in mitochondria and the chloroplasts, respectively, fits the model of separate assembly sites in mitochondria and chloroplasts. However, why AtISU2 mRNA and protein is not accumulated at high levels in the chloroplasts remains unknown. The reason for the lack of activation of AtISU3 could be that in the mitochondrial machinery, AtISU1 is the predominant scaffold protein and AtISU3 maybe an inducible one.

Antisense and RNAi technologies are powerful tools for studying the effects of down regulation of gene expression that can give insights into the physiological roles of the targeted genes. Down-regulation of the *AtISU* genes using these strategies exhibited several striking phenotypes consistent with effects on hormone biosynthesis, energy production, and primary metabolism, all of which depend on functional [Fe-S] proteins. While RNAi is capable of complete gene silencing, antisense suppression usually leads to gradations of silencing. Antisense AtISU1 plants grew well but were

bushy in appearance, showed loss of apical dominance as well as showed appearance of clusters of flowers at the base of the stem. However, it should be noted that due to the high sequence similarity of the *AtISU* genes, an antisense construct for AtISU1 could also cause silencing of *AtISU2* and *AtISU3.*

The effects seen for the AtISU2 and AtISU3 RNAi plants were more dramatic as could be expected due to a higher degree or complete silencing. These plants were not as robust as wild type plants. A partial or complete rosette pattern was repeated multiple times higher in the shoot and the cauline leaves were considerably larger than those of the wild type plants in both cases. Further, an obvious perturbation in the rosette pattern with the appearance of large leaves was observed in some of the AtISU2 RNAi plants. Some AtISU2 RNAi plants also showed a distinct early onset of bolting. Although a precise physiological effect cannot be assigned to each of these phenotypes at this point, these observations suggest that AtISU2 and AtISU3 are important for plant development. To obtain an initial overview of the proteins affected in the antisense and RNAi lines, two-dimensional-gel analysis could be used to identify abundant proteins that are destabilized in the absence of an [Fe-S] prosthetic group. Identification of the specific proteins affected by down regulation of individual AtISU isoforms may help determine whether specific target proteins depend on specific assembly systems. Further, considering the possibility that AtISU3 is inducible, it will also be interesting to examine the effect of AtISU3 RNAi constructs in plants that are grown under different conditions. These experiments could determine whether the controlled distribution of [Fe-S] clusters is a method of regulation in plants.

The phenotypes of the three AtISU down-regulated plants showed some similarities which included aberrant development of inflorescence, rosettes, cauline leaves, and shoots. It also showed that these proteins were dispensable, however, the severe phenotypes observed indicated that the functions of the alternate scaffold proteins cannot completely compensate for each other, and that a true difference in the complement of target apoproteins may exist for each AtISU isoform. This complex network could perhaps be understood by generating multiple knockouts in one plant.

In comparison to the two sulfur-donors, AtNFS1 and AtNFS2, there are a baffling number of potential scaffold proteins in plants including AtISU1-3, AtNFU1-5, and AtISA1-4. Why are there so many scaffold proteins? Would then the expression of AtISU3 under specific conditions really be that special? These questions still remain open. The field of [Fe-S] cluster assembly continuous to evolve rapidly. Characterizing the potentially diverse functions of each these proteins may help define the relationship between the two systems and further increase understanding of the adaptations that plants have evolved for survival.

MATERIALS AND METHODS

DNA and protein sequence analysis

Sequence analysis and phylogenetic tree construction were performed using the Lasergene suite of DNA analysis programs (DNASTAR, Madison, WI). Analysis of targeting sequences was performed using PSORT (http://psort.nibb.ac.jp/) and TargetP (http://www.cbs.dtu.dk/services/TargetP/). Protein sequence comparisons were made using the following sequences: *Azotobacter* IscU (accession number AAC24473), and

NifU (AAA2216), *Arabidopsis* AtISU1 (15235569), AtISU2 (15233385), AtISU3 (15232028), *Homo sapiens* ISCU1 (AAG37427), *Homo sapiens* ISCU2 (AAG37428), *Mus musculus* ISCU (BAB26031), *Drosophila melanogaster* (AAF54298), *Haemophilus influenza* (ISCU), *Pasteurella multocida* (ISCU), *E. coli* IscU (BAB36818), *S. cerevisiae* Isu1p (NP015190), and Isu2p (NP014869).

Plants and Growth Conditions

Arabidopsis thaliana ecotype Columbia was used in these studies. Seeds were grown on Murashige Skoog (MS) sucrose agar (Gibco, BRL) as described previously (Kubasek et al., 1992) under 120 $\mu mol^{-1}m^{-2}$ light intensity. Briefly, the seeds were surface sterilized by soaking in 100% ethanol for 1 min followed by 3% NaOCl, 0.05% Triton X-100 for 5 min, followed by three rinses in sterile distilled water. The seeds were then suspended in 2 ml 0.15% agar and spread on MS medium, pH 5.7, containing 2% sucrose and 0.8% agar. The plates were sealed with Nescofilm® (Karlan), wrapped in aluminum foil, and the seeds were vernalized for 2-4 days at 4°C. The plates were then incubated at 22°C under continuous light for approximately 10 days. The seedlings were transferred to soil and grown at 22 ^{0}C under a 16 h/8 h light/dark cycle.

Plant Transformation

The freeze-thaw method (Chen et al., 1994) was used to transform *Agrobacterium tumefaciens* strain GV3101 with the various constructs. *Arabidopsis* ecotype Columbia plants were transformed using the floral dip method (Clough and

Bent, 1998) for the pBW18 and pCAMBIA3300-derived constructs while for the pCAMBIA1302 and pBIB-derived constructs, the floral dip method was followed by vacuum infiltration. Transgenic plants were selected on MS-sucrose plates supplemented with 100 μg/ml kanamycin or 100 μg/ml hygromycin or by spraying with glufosinate ammonium (phosphinothricin, Basta®/Liberty) herbicide (AgrEvo Company, Wilmington, DE, USA). The final concentration of the Basta® solution used for spraying on plants contained 1:10000 dilution of the Basta® concentrate and 0.005% Silwet L-77 (Lehle Seeds, Round Rock, TX) as per the manufacturer's instructions.

The seeds were vernalized in darkness at 4°C (on plates or soil) for 2 days and then transferred to constant white light at 22 °C. For kanamycin or hygromycin selection, seedlings that were green after 10 days of growth were transferred to soil and grown at 22 °C under a 16 h/8 h light/dark cycle. In the case of Basta® resistance, seedlings growing in soil were sprayed with the Basta® herbicide starting at approximately 10 days of age for three days in succession with a 4 day gap (a total of six days of spraying). Strong survivors were transplanted to fresh soil and used for further analysis. Second generation (T2), third generation (T3), and fourth generation (T4) seeds were collected and transgenics confirmed by growth on MS sucrose plates containing the selective antibiotic or by Basta® spraying to follow segregation of the transgene and identify homozygous lines.

Microscopy

Light microscopy was performed according to standard protocols using a Stemi SV11 Apo microscope (Zeiss Diagnostic Instruments, USA) equipped with a KY-F70

Victor, JVC camera and an Olympus SZX12 microscope . For CLSM microscopy, fully expanded rosette leaves from 3-4 week old plants were mounted in water under cover slips. Specimens were examined with an 40X C-Apochromat objective lens (water corrected, numerical aperture 1.2) using a LSM 510 (Carl Zeiss Inc., Thornwood, NY). Fluorescent images were obtained up to 532 X 512 pixel resolution by using the 488 nm excitation line of an argon laser for mGFP fluorescence and a helium-neon laser (633 nm) for chlorophyll autofluorescence, with 505- to 530- nm band pass (BP) and 650 nm long pass (LP) filters for GFP and chlorophyll, respectively. The samples were optically sectioned up to 1 µm to reconstruct 3-D images with the software package supplied with the microscope. Differential interference contrast (DIC) reference images were also collected. Images were exported as JPEG files and further processed with Adobe Photoshop (version 5.02; Adobe Systems, San Jose, CA).

AtISU::mGFP constructs

In order to determine the subcellular localization of the AtISU1 protein, AtISU1::mGFP constructs were generated using the BAC clone, K3F7TP (obtained from Arabidopsis Biological Research Center, Columbus, OH), as a template (Ana Frazzon, unpublished). The *AtISU1* coding region without the stop codon (165 aa), was amplified using the forward primer, 5'-CATGCCATGGGTATGATGCTCAAGCAAGCTG-3' adding a *Nco*I site at the 5' end together with 5'-CATGCCATGGCCTGTGTGGTTTCTCC-3' as reverse primer. The resulting fragment was digested with *Nco*I and cloned in-frame with the amino terminus of mGFP5 in pAVA393 (von Arnim et al., 1998). The AtISU1::GFP including the double-enhanced cauliflower mosaic virus CaMV 35S promoter, Tobacco

Etch Virus (TEV) translational leader, and nopaline synthase (nos) terminator sequences, were excised with *Sal*I and *Xma*I and cloned into the corresponding sites in the binary vector, pBIB (Becker, 1990a). The integrity of the AtISU1::GFP construct was tested by examining GFP fluorescence by CLSM following particle bombardment into onion epidermal cells (Scott et al., 1999). In the case of *AtISU2* and *AtISU3*, the coding regions including the introns but without the stop codon were amplified from genomic DNA (Watson and Thompson, 1986) with *Nco*I linkers, cloned in-frame at the *Nco*I site at the amino terminus of the plant-optimized reporter gene, *mGFP5*, under the control of the double-enhanced CaMV 35S promoter in the plant binary vector, pCAMBIA1302 (Cambia, Canberra, Australia), and expressed in transgenic *Arabidopsis* ecotype Columbia plants. A schematic of the constructs is shown in Figure 12. Primers used to amplify the inserts are listed in Table 2.

AtISU1, AtISU2 and *AtISU2* promoter::GUS constructs

To examine the pattern of *AtISU1-3* promoter activity, the putative 5' regulatory sequences of the *AtISU1-3* genes were placed upstream of the promoter-less GUS reporter gene in the binary vector, pBW18 (gift of Bonnie Woffenden, McDowell Lab, Virginia Tech), which was made from pCAMBIA1391Z by removing the *Xho*I fragment containing the hygromycin resistance gene and replacing it with the *Xho*I fragment containing the Bar gene from pCAMBIA3300. A schematic of the constructs is shown in Figure 13. Primers with *Nco*I linkers (Table 3) were used to amplify the 5' sequences from genomic DNA with *Pfu* polymerase (Stratagene, La Jolla, CA). For screening purposes these same sequences or the *GUS* gene were amplified using the forward

Figure 12. Schematic representation of the AtISU::GFP constructs used for localization studies in transgenic *Arabidopsis*. The *AtISU* genes were amplified without the stop codon; *Nco*I linkers were introduced using primers. Amplified fragments were cloned into the *Nco*I site of the pCAMBIA1302 vector.

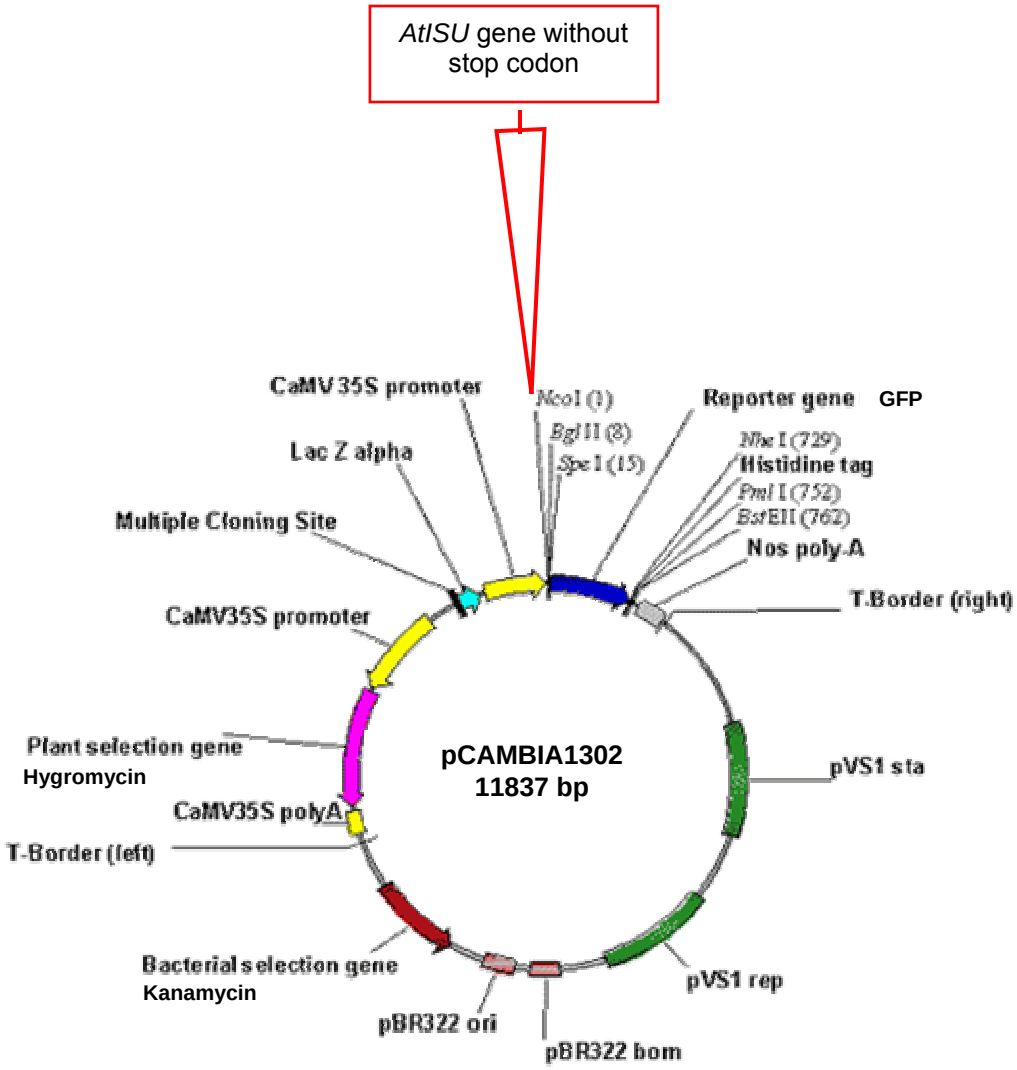


Table 2. Primers containing *Nco*I linkers used in amplifying *AtISU2* and *AtISU3* genes for cloning into the pCAMBIA1302 vector. Gene sequences are underlined.

Gene	Primer direction	Primer Sequence
AtISU2	Forward	5' CATGCCATGG GTATGATGATGCTAAGGCAAAC 3'
	Reverse	5' CATGCCATGG CCTTGACGGTTTCTTC 3'
AtISU3	Forward	5' CATGCCATGG GTATGTTAAGGCAAACAACTAAAAG 3'
	Reverse	5' CATGCCATGG CGATCCAATACCGTGTAAATAAG 3'

Figure 13. Schematic of the *AtISU* Promoter::GUS constructs. Upstream regions of the *AtISU* genes, including the 5' UTR, were cloned into the plant binary vector, pBW18, in-frame with the amino terminus of the β-glucuronidase GUS reporter gene. These regions were 1400 bp, 968 bp, and 340 bp for the *AtISU1*, *AtISU2*, and *AtISU3* genes respectively.

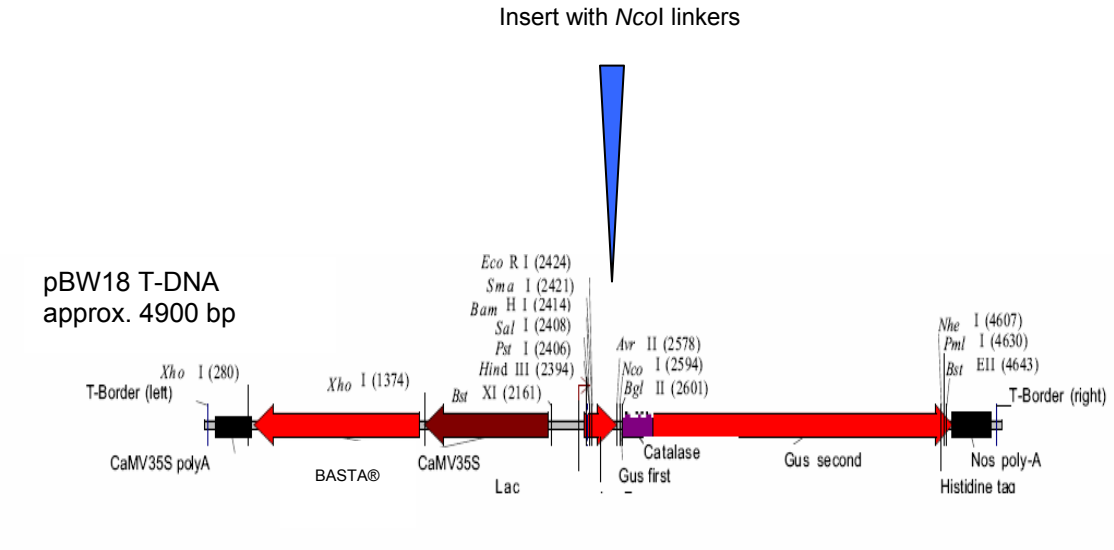


Table 3. Primers containing *Nco*I linkers used for amplifying the 5' regulatory regions of the *AtISU* genes for cloning into the pBW18 vector. Gene sequences are underlined.

Promoter	Primer direction	Primer Sequence
AtISU1	Forward	5' CATGCCATGGTAATTGCTTATTAGTTCTTAGCTTC 3'
	Reverse	5' CATGCCATGGCGTGTTTTTTTTAGGGTTTCGG 3'
AtISU2	Forward	5' CATGCCATGGGAGCACAGTGGAGAC 3'
	Reverse	5' CATGCCATGGATCGATCGCTTCTCTC 3'
AtISU3	Forward	5' CATGCCATGGCTCAGACTCTTTGCC 3'
	Reverse	5' CATGCCATGGATTCTCTCTTTCTCTTTG 3'

primer, 5'-CCCAGGCTTTACACTTTATGC-3', and the reverse primer, 5'-CAGGACGGACGAGTCGTCGG-3' with *Taq* polymerase (Stratagene, La Jolla, CA).

AtISU1 sense and antisense constructs

To study the effect of overexpression and suppression of *AtISU1*, sense and antisense constructs were generated by Ana Frazzon (unpublished). Briefly, *AtISU1* sequences including the stop codon were amplified from the cDNA clone K3F7TP using the forward primer, 5'-CATGCCATGGCCTGTGTGGTTTCTCC-3', the reverse primer, 5'-CATGCCATGGTCAAGCCTGTGTGGTTTC-3', and *Pfu* polymerase. The resulting 503 bp fragment was digested with *Nco*I and cloned into the corresponding site in pBLRTL2 (Chapter 2). The orientation of the inserts was determined by PCR and restriction enzyme analysis. The resulting constructs were excised with *Sal*I and *Kpn*I and cloned into the corresponding sites in pBIB (Becker, 1990b). This construct was used to transform *A. tumefaciens* strain GV3101 as described above, which was then used to transform plants.

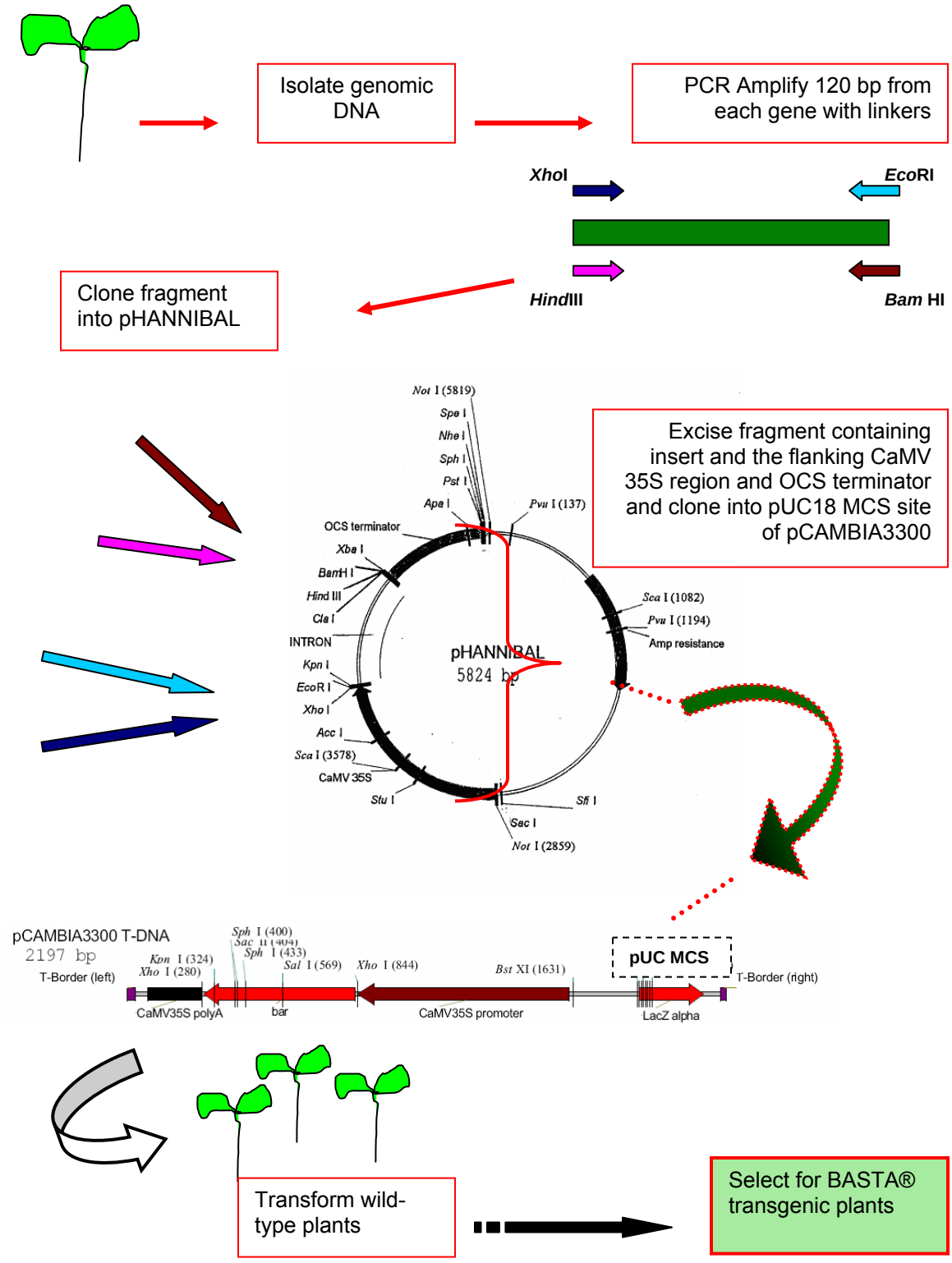
AtISU1, AtISU2 and *AtISU2* RNAi constructs for expression in plants

In order to silence the AtISU genes individually, 120 bp fragments at the 3' end (83-85 bp into the UTR) were amplified with linkers (Table 4) and cloned in the sense and antisense orientations in the pHANNIBAL cloning vector (CSIRO, Australia and Wesley et al., 2001). A schematic of the RNAi constructs is shown in Figure 14. For AtISU2 and the pHANNIBAL empty vector control, the cassette including the double enhanced CaMV promoter and the nopaline synthase terminator were subcloned into

Table 4. Primers used in the generation of RNAi fragments for the *AtISU* genes for cloning into the pHANNIBAL vector. Gene sequences are underlined.

Gene	Orientation	Primer direction	Linker	Primer Sequence
AtISU1	Sense	Forward	XhoI	5' CCGCTCGAG GTGCTGCAGCAGCAGG 3'
		Reverse	EcoRI	5' CCGGAATTC ATAGGTTGTATGTATCATCC 3'
	Antisense	Forward	HindIII	5' CCCAAGCTT ATAGGTTGTATGTATCATCC 3'
		Reverse	BamHI	5' CGCGGATCC GTGCTGCAGCAGCAGG 3'
AtISU2	Sense	Forward	XhoI	5' CCGCTCGAG TCGATTCCACTTATTTACAC 3'
		Reverse	EcoRI	5' CCGGAATTC ACAAGACTTGAGTAAAACTC 3'
	Antisense	Forward	HindIII	5' CCCAAGCTT ACAAGACTTGAGTAAAACTC 3'
		Reverse	BamHI	5' CGCGGATCC TCGATTCCACTTATTTACAC 3'
AtISU3	Sense	Forward	XhoI	5' CCGCTCGAG CTGCTGCTGCAGAAGAAACCG 3'
		Reverse	EcoRI	5' CCGGAATTC GAAAAACTAAAACAACCAAAC 3'
	Antisense	Forward	HindIII	5' CCCAAGCTT GAAAAACTAAAACAACCAAAC 3'
		Reverse	BamHI	5' CGCGGATCC TCGATTCCACTTATTTACAC 3'

Figure 14. Schematic of the constructs used for the generation of AtISU1, AtISU2, and AtISU3 RNAi plants.



the pCAMBIA3300 vector using *Sac*I and *Pst*I. AtISU1 and AtISU3 RNAi cassettes were subcloned into the *Sma*I site of pCAMBIA3300 using *Not*I. Constructs containing the RNAi cassettes were used to transform *A. tumefaciens* strain GV3101, which was then used to transform plants.

REFERENCES

Agar, J.N., Zheng, L., Cash, V.L., Dean, D.R., and Johnson, M.K. (2000a). Role of the IscU protein in iron-sulfur cluster biosynthesis: IscS-mediated assembly of a [Fe_2S_2] cluster in IscU. JACS Comm.**,** 2136-2137.

Agar, J.N., Krebs, C., Frazzon, J., Huyhn, 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] cluster in IscU. Biochemistry **39,** 7856-7862.

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

Alonso, J., Stepanova, A., Leisse, T., Kim, C., Chen, H., Shinn, P., Stevenson, D., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W., Berry, C., and Ecker, J. (2003). Genome-Wide Insertional Mutagenesis of Arabidopsis thaliana. Science **301,** 653-657.

Becker, D. (1990a). Binary vectors which allow the exchange of plant selectable markers and reporter genes. Nucl. Acids. Res. **18,** 203-210.

Becker, D. (1990b). Binary vectors which allow the exchange of plant selectable markers and reporter genes. Nucleic Acids Res. **18,** 203.

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

Bekri, S., Kispal, G., Lange, H., Fitzimonis, E., Tolmie, J., Lill, R., and Bishop, D.F. (2000). Human ABC7 transporter: gene structure and mutation causing X-linked sideroblastic anemia with ataxia with disruption of cytosolic iron-sulfur protein maturation. Blood **9,** 3256-3264.

Chen, H., Nelson, R.S., and Sherwood, J.L. (1994). Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. Biotechniques **16,** 664-668, 670.

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

Craig, E.A., Voisine, C., and Schilke, B. (1999). Mitochondrial iron metabolism in the yeast Saccharomyces cerevisiae, **Biol Chem,** 1167-1173.

Csere, P., Lill, R., and Kispal, G. (1998). Identification of a human mitochondrial ABC transporter, the functional orthologue of yeast Atm1p. FEBS Lett **441,** 266-270.

Dutkiewicz, R., Schilke, B., Knieszner, H., Walter, W., Craig, E.A., and Marszalek, J. (2003). Ssq1, a mitochondrial Hsp70 involved in iron-sulfur (Fe/S) center biogenesis. Similarities to and differences from its bacterial counterpart. J. Biol. Chem. **278,** 29719-29727.

Ellis, K.E., Clough, B., Saldanha, J.W., and Wilson, R.J. (2001). NifS and SufS in malaria. Mol. Microbiol. **41,** 973-981.

Flint, D.H. (1996). *Escherichia coli* contains a protein that is homologous in function and N-terminal sequence to the protein encoded by the *nifS* gene of *Azotobacter vinelandii* and that can participate in the synthesis of the Fe-S cluster of dihydroxy-acid dehydratase. J. Biol. Chem. **271,** 16068-16074.

Frazzon, J., and Dean, D.R. (2001). Feedback regulation of iron-sulfur cluster biosynthesis. Proc Natl Acad Sci U S A **98,** 14751-14753.

Frazzon, J., and Dean, D.R. (2003). Formation of iron-sulfur clusters in bacteria: an emerging field in bioinorganic chemistry. Curr. Op. Chem. Biol. **7,** 166-173.

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

Garland, S.A., Hoff, K., Vickery, L.E., and Culotta, V.C. (1999). Saccharomyces cerevisiae ISU1 and ISU2: members of a well-conserved gene family for iron-sulfur cluster assembly. J. Mol. Biol. **294,** 897-907.

Gerber, J., and Lill, R. (2002). Biogenesis of iron-sulfur proteins in eukaryotes: components, mechanism and pathology. Mitochondrion **2,** 71-86.

Gerber, J., Mühlenhoff, U., and Lill, R. (2003). An interaction between frataxin and Isu1/Nfs1 that is crucial for Fe/S cluster synthesis on Isu1. EMBO J. **4,** 906-911.

Haseloff, J., Siemering, K.R., Prasher, D.C., and Hodge, S. (1997). Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc Natl Acad Sci U S A **94,** 2122-2127.

Jaschkowitz, K., and Seidler, A. (2000). Role of a NifS-like protein from the cyanobacterium *Synechocystis* PCC 6803 in the maturation of FeS proteins. Biochem. **39,** 3416-3423.

Kato, S., Mihara, H., Kurihara, T., Yoshimura, T., and 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., and 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.

Kiley, P.J., and Beinert, H. (2003). The role of Fe-S proteins in sensing and regulation in bacteria. Curr. Opin. Microbiol. **6,** 181-185.

Kispal, G., Csere, P., Prohl, C., and Lill, R. (1999). The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. EMBO J. **18,** 3981-3939.

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.

Kubasek, W.L., Shirley, B.W., McKillop, A., Goodman, H.M., Briggs, W., and Ausubel, F.M. (1992). Regulation of flavonoid biosynthetic genes in germinating Arabidopsis seedlings. Plant Cell **4,** 1229-1236.

Kurihara, T., Mihara, H., Kato, S., Yoshimura, T., and Esaki, N. (2003). Assembly of iron-sulfur clusters mediated by cysteine desulfurases, IscS, CsdB and CSD, from *Escherichia coli*. Biochim. Biophys. Acta. **1647,** 303-309.

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.

Lange, H., Kaut, A., Kispal, G., and Lill, R. (2000). A mitochondrial ferredoxin is essential for biogenesis of celllular iron-sulfur proteins. Proc Natl Acad Sci U S A **97,** 1050-1055.

Lange, H., Lisowsky, T., Gerber, J., Muhlenhoff, U., Kispal, G., and Lill, R. (2001). An essential function of the mitochondrial sulfhydryl oxidase Erv1p/ALR in the maturation of cytosolic Fe/S proteins. EMBO Rep. **2,** 715-720.

Leibrecht, I., and Kessler, D. (1997). A novel L-cysteine/cystine C-S-lyase directing [2Fe-2S] cluster formation of *Synechocystis* ferredoxin. J. Biol. Chem. **272,** 10442-10447.

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

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

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

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

Lill, R., Diekert, K., Kaut, A., Lange, H., Pelzer, W., Prohl, C., and Kispal, G. (1999). The essential role of mitochondria in the biogenesis of cellular iron-sulfur proteins. Biol Chem. **380,** 1157-1166.

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.

Masepohl, B., Angermuller, S., Hennecke, S., Hubner, P., Moreno-Vivian, C., and Klipp, W. (1993). Nucleotide sequence and genetic analysis of the *Rhodobacter capsulatus* ORF6-*nifUI* SVW gene region: possible role of *NifW* in homocitrate processing. Mol. Gen. Genet. **238,** 369-382.

Merchant, S., and Dreyfuss, B.W. (1998). Posttranslational assembly of photosyntheic metalloproteins. Annu Rev Plant Physiol Plant Mol Biol **49,** 25-51.

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

Mühlenhoff, U., and Lill, R. (2000). Biogenesis of iron-sulfur proteins in eukaryotes: a novel task of mitochondria that is inherited from bacteria. Biochim. Biophys. Acta. **1459,** 370-382.

Mühlenhoff, U., Richhardt, N., Gerber, J., and Lill, R. (2002). Characterization of iron-sulfur protein assembly in isolated mitochondria. J. Biol. Chem. **277,** 29810–29816.

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

Nakamura, M., Saeki, K., and Takahashi, Y. (1999). Hyperproduction of recombinant ferredoxins in escherichia coli by coexpression of the ORF1-ORF2-iscS-iscU-iscA-hscB-hs cA-fdx-ORF3 gene cluster. J. Biochem. (Tokyo) **126,** 10-18.

Nakamura, T., Yamaguchi, Y., and Sano, H. (2000). Plant mercaptopyruvate sulfurtransferases. Molecular cloning, subcellular localization and enzymatic activities. Eur. J. Biochem. **267,** 5621-5630.

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

Ollagnier-de-Choudens, S., Mattioli, T., Takahashi, Y., and 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.

Ouzounis, C., Bork, P., and Sander, C. (1994). The modular structure of NifU proteins. Trends Biochem. Sci. **19,** 199-200.

Papenbrock, J., and Schmidt, A. (2000). Characterization of a sulfurtransferase from *Arabidopsis thaliana*. Eur. J. Biochem. **267,** 145-154.

Patzer, S.I., and Hantke, K. (1999). SufS is a NifS-like protein, and SufD is necessary for stability of the [2Fe-2S] FhuF protein in *Escherichia coli*. J. Bacteriol. **181,** 3307-3309.

Pelzer, W., Mühlenhoff, U., Diekert, K., Siegmend, K., Kispal, G., and Lill, R. (2000). Mitochondrial Isa2p plays a crucial role in the maturation of iron-sulfur proteins. FEBS Lett. **476,** 134-139.

Pilon-Smits, E.A., 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.

Roy, A., Solodovnikova, N., Nicholson, T., Antholine, W., and Wailden, W. (2003). A novel eukaryotic factor for cytosolic Fe-S cluster assembly. EMBO J. **22,** 4826-4835.

Schilke, B., Voisine, C., Beinert, H., and Craig, E. (1999). Evidence for a conserved system for iron metabolism in the mitochondria of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **96,** 10206-10211.

Scott, A., Wyatt, S., Tsou, P.L., Robertson, D., and Allen, N.S. (1999). Model system for plant cell biology: GFP imaging in living onion epidermal cells. Biotechniques **6,** 1128-1132.

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.

Strain, J., Lorence, C.R., Bode, J., Garland, S.A., A., S.G., Ta, D.T., Vickery, L.E., and Culotta, V.C. (1998). Suppressors of superoxide dismutase (SOD1) deficiency in Saccharomyces cerevisiae. Identification of proteins predicted to mediate iron-sulfur cluster assembly. J. Biol. Chem. **273,** 31138-31144.

Tachezy, J., Sanchez, L.B., and Muller, M. (2001). Mitochondrial type iron-sulfur cluster assembly in the amitochondriate eukaryotes *Trichomonas vaginalis* and *Giardia intestinalis*, as indicated by the phylogeny of IscS. Mol. Biol. Evol., 1919-1928.

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 homolgs in Archaea and plastids. J. Biol. Chem. **277,** 28380-28383.

Takahashi, Y., Mitsui, A., and Matsubara, H. (1991). Formation of the Fe-S cluster of ferredoxin in lysed spinach chloroplasts. Plant Physiol. **95,** 97-103.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22,** 4673-4680.

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

Tokumoto, U., Nomura, S., Minami, Y., Mihara, H., Kato, S., Kurihara, T., Esaki, N., Kanazawa, H., Matsubara, H., and Takahashi, Y. (2002). Network of protein-protein interactions among iron-sulfur cluster assembly proteins in *Escherichia coli*. J. Biochem. **131,** 713-719.

Tong, W.H., and Rouault, T. (2000). Distinct iron-sulfur cluster assembly complexes exist in the cytosol and mitochondria of human cells. EMBO J. **19,** 5692-5700.

Tong, W.H., Jameson, G.N., Huynh, B.H., and Rouault, T.A. (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 U S A **100,** 9762-9767.

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.

von Arnim, A.G., Deng, X.W., and Stacey, M.G. (1998). Cloning vectors for the expression of green fluorescent protein fusion proteins in transgenic plants. Gene **221,** 35-43.

Watson, J.C., and Thompson, W.F. (1986). Purification and restriction endonuclease analysis of plant nuclear DNA. Methods Enzymol. **118,** 57-75.

Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M.B., Rouse, D.T., Liu, Q., Gooding, P.S., Singh, S.P., Abbott, D., Stoutjesdijk, P.A., Robinson, S.P., Gleave, A.P., Green, A.G., and Waterhouse, P.M. (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J. **27,** 581-590.

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., White, R.H., Cash, V.L., and Dean, D.R. (1994). Mechanism for the desulfurization of L–cysteine catalyzed by the *nifS* gene product. Biochemistry **33,** 4714–4720.

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.

CHAPTER 4

SUMMARY

Iron is the most abundant transition element in the earth's crust and sulfur among the most versatile. In nature, these two elements are often combined into clusters of which $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ are the most common types. The structure of these clusters is dynamic and has electronic properties which may have been exploited in the primordial soup. All major biological systems contain proteins that use [Fe-S] clusters as cofactors and these clusters are fundamental to life as it exists now. For example, electron acceptors, environmental sensors, and some proteins in photosynthesis, respiration, and nitrogen fixation contain [Fe-S] clusters. Many of these processes would not function in the absence of these small, simple, inorganic molecules. The use of these clusters has, however, been challenging to cells because of the toxicity of free Fe and S. Living organisms appear early on to have evolved controlled mechanisms for the assembly of these clusters. An ancient mechanism of [Fe-S] cluster assembly that is highly conserved with regard to the basic features of the players, their functions, and their primary sequences appears to be present in the vast majority of present-day microorganisms, animals, and plants (reviewed in Beinert, 2000; Gerber and Lill, 2002; Frazzon and Dean, 2003; Kiley and Beinert, 2003).

Identification of the iron sulfur cluster (*isc*) operon that orchestrates [Fe-S] cluster assembly in *Azotobacter vinelandii* is among the most important discoveries in biology in the past twelve years. The *isc* operon is highly homologous to part of the *nif* operon, which encodes nitrogenase, the major enzyme of nitrogen fixation in bacteria. In addition to the *nif* and *isc* operons, another system, designated *suf* for its role in sulfur mobilization, has been found to play a role in [Fe-S] cluster formation under iron depletion and oxidative stress conditions in *Escherichia coli* (Patzer and Hantke, 1999;

Ellis et al., 2001; Takahashi and Tokumoto, 2002). However, not all organisms contain a complete set of the nif, isc, or suf machinery and evidence exists that suggests the existence of additional [Fe-S] biogenesis mechanism(s) (Leibrecht and Kessler, 1997; Jaschkowitz and Seidler, 2000; Kato et al., 2000). In eukaryotes, most information has been derived from studies in yeast in which the [Fe-S] cluster assembly machinery has been found to be compartmentalized within mitochondria, from which these clusters are transported to the cytoplasm (Lill and Kispal, 2000). In humans, [Fe-S] clusters are also found to be assembled in the nucleus and cytoplasm (Tong and Rouault, 2000). Not much information is available on this mechanism in plants, which provide an extra challenge because [Fe-S] cluster assembly was shown to also occur in chloroplasts (Léon et al., 2002b; Pilon-Smits et al., 2002; Léon et al., 2003).

The IscS- and IscU-like proteins are the major players in the assembly of [Fe-S] clusters. IscS catalyzes the release of elemental sulfur from L-cysteine to provide the inorganic sulfur necessary for [Fe-S] cluster formation (Zheng et al., 1993). IscU is a scaffold protein on which the transient [Fe-S] cluster is assembled by a mechanism that is not yet entirely clear (Agar et al., 2000). Moreover, the details of how clusters are delivered to and inserted into target proteins also remain unknown.

The objective of the study described in this dissertation has been to characterize several candidates that are major players in the [Fe-S] cluster assembly mechanism in plants, using *Arabidopsis thaliana* as a model system. The project focused on four largely uncharacterized genes, AtNFS1, AtISU1, AtISU2, and AtISU3. AtNFS1 has 54.7 % amino acid identity to *A. vinelandii* IscS and the N-terminal 54 amino acids of this protein have been shown to target GFP to mitochondria (Kushnir et al., 2001). The

AtISU1-3 proteins have 65.6-67.2 % amino acid identity with *A. vinelandii* IscU. T-DNA insertional mutants have been identified for AtNFS1, AtISU2, and AtISU3. It is not known if these mutant lines are homozygous. Loss of AtISU2 or AtISU3 activity in these knockouts may be compensated for by the overlapping functions of AtISU1, and members of two other families of potential scaffold proteins, ATNFU1-5 and AtISA1-4. However, it would be surprising if AtNFS1 is dispensable considering that there is no other IscS homolog present in mitochondria.

Work done previously on this project has shown that AtNFS1 can function as a cysteine desulfurase and assemble transient [Fe-S] clusters *in vitro* in IscU and AtISU1. It has now also been shown that full-length AtNFS1 is localized exclusively in mitochondria, and transgenic plants expressing *AtNFS1* in the antisense orientation exhibit phenotypes consistent with a role for this gene in hormone biosynthesis, the response to oxidative stress, and sulfur assimilation (Chapter 2; Ana Frazzon, unpublished). These results provide direct evidence for a [Fe-S] cluster assembly system in plant mitochondria. The putative scaffold proteins, AtISU1 and AtISU3, were also found to localize to mitochondria while AtISU2 localizes to chloroplasts. The promoters of AtISU1 and AtISU2 appear to be constitutively active while AtISU3 promoter activity was not detected in any tissues of healthy, unstressed plants, regardless of age. Interestingly, the EST database contains numerous sequences for AtISU1 but none for AtISU2 or AtISU3. Also, transgenic AtISU2::GFP plants did not show any accumulation of the recombinant protein while numerous high-expressing lines were identified for AtISU1::GFP and AtISU3::GFP. AtISU2::GFP did however show GFP activity. Together, these findings indicate that AtISU2 mRNA is unstable.

Thus the genes encoding the three AtISU isoforms are differentially regulated at the transcriptional and perhaps also at the posttranscriptional level.

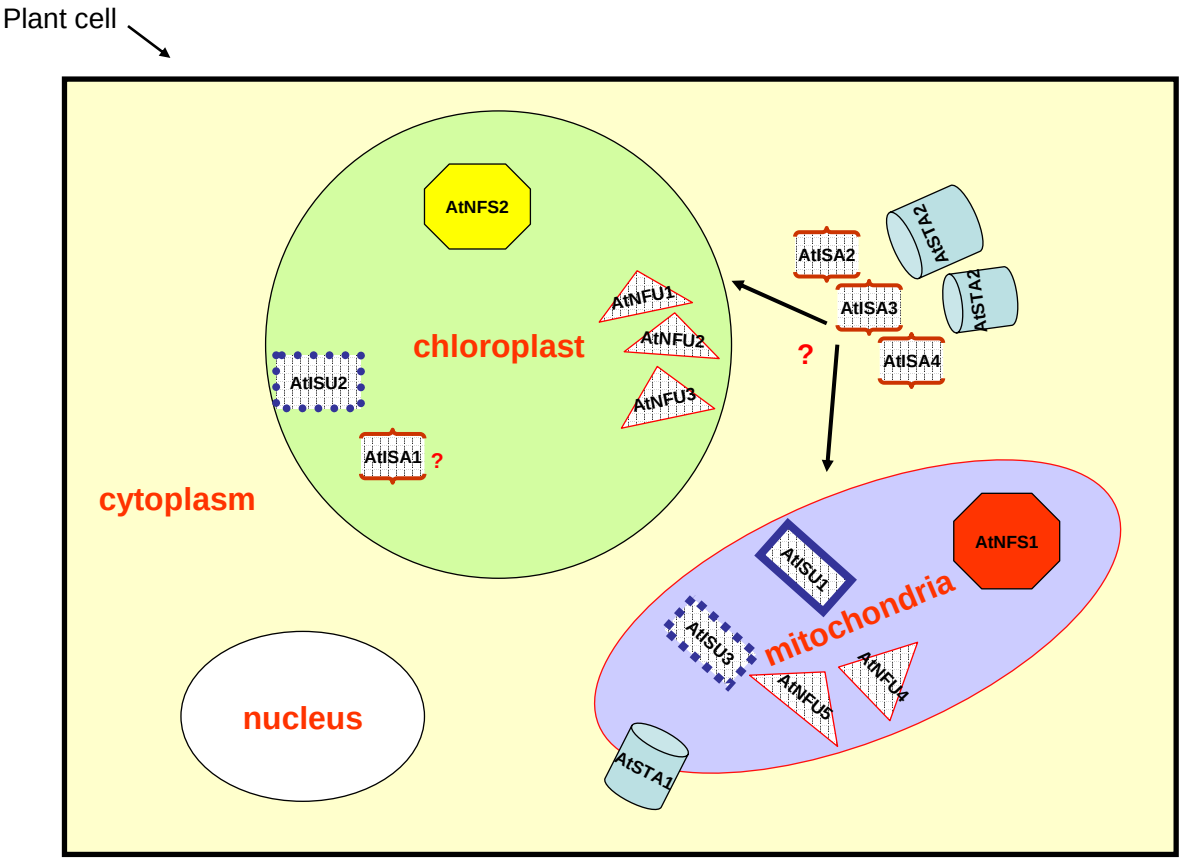
Down regulation of each of the AtISU genes and AtNFS1 using antisense or RNAi technology resulted in phenotypes consistent with a role for these genes in hormone biosynthesis, energy production, and primary metabolism. AtNFS1 down regulation showed severe phenotypes and also appeared to have an effect on genes involved in photosynthesis. It is unlikely that an AtNFS1 knock could survive considering it is the only known IscS homolog in mitochondria. It may be possible that AtNFS2 can partially replace some functions of AtNFS1. These observations, together with previous *in vitro* evidence that AtISU1 and AtNFS1 can participate in [Fe-S] cluster assembly (Chapter 2; Ana Frazzon, unpublished), provide strong evidence that AtNFS1 and AtISU1-3 participate in [Fe-S] cluster assembly in different subcellular locations and perhaps under different environmental conditions.

In addition to AtNFS1 and AtISU1-3, other genes such as AtNFS2 which show sequence similarity to the SufS (Léon et al., 2002a; Pilon-Smits et al., 2002), and AtNFU1-5 which show sequence similarity to the C-terminus of NifU (Léon et al., 2003), have been identified. AtNFU4 localizes to mitochondria while AtNFS2 and AtNFU1-3 localize to chloroplasts. Furthermore, AtSTA1 has been identified as a mitochondrial ABC transporter that plays a role in the mobilization of [Fe-S] clusters to the cytoplasm. The locations of two other homologs, AtSTA2 and AtSTA3, remains to be established (Kushnir et al., 2001). Thus AtNFS1, AtISU1, AtISU3, AtNFU4, AtNFU5, and AtSTA1 appear to be part of a mitochondrial assembly system analogous to the yeast

machinery, while AtNFS2, AtISU2, and AtNFU1-3 are part of a system located in chloroplasts (Figure 1).

Many basic questions still remain open regarding the [Fe-S] cluster assembly mechanism in plants, such as why there are so many scaffold proteins relative to bacteria or yeast and why plant cells need two sites for [Fe-S] cluster assembly. The controlled distribution of [Fe-S] clusters to distinct subsets of target proteins located in diverse cellular locations could be one mechanism of regulation of [Fe-S] protein activities. Characterizing the specific functions of AtNFS1 and AtISU1-3 and defining the relationships between the mitochondrial and chloroplast systems will help in understanding how this machinery has evolved to serve the unique requirements of the plant cell. This information could be helpful from the perspective of metabolic engineering to enhance plant productivity by modifying the distribution of active [Fe-S] proteins.

Figure 1. Schematic model of proteins known to be involved in [Fe-S] cluster biogenesis in plants. NifU/IscU homologs: AtISU1, AtISU2, AtISU3, AtISA, AtISA2, AtISA3, AtISA4, AtNFU1, AtNFU2, AtNFU3, AtNFU4, and AtNFU5; NifS/IscS homologs: AtNFS1 and ANFS2; ABC transporters: AtSTA1, AtSTA2, and AtSTA3.



REFERENCES

Agar, J.N., Krebs, C., Frazzon, J., Huyhn, B.H., Dean, D.R., and Johnson, M.K. (2000). IscU as a scaffold for iron-sulfur cluster biosynthesis: Sequential assembly of [2Fe-2S] and [4Fe-4S] cluster 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.

Ellis, K.E., Clough, B., Saldanha, J.W., and Wilson, R.J. (2001). NifS and SufS in malaria. Mol. Microbiol. **41,** 973-981.

Frazzon, J., and Dean, D.R. (2003). Formation of iron-sulfur clusters in bacteria: an emerging field in bioinorganic chemistry. Curr. Op. Chem. Biol. **7,** 166-173.

Gerber, J., and Lill, R. (2002). Biogenesis of iron-sulfur proteins in eukaryotes: components, mechanism and pathology. Mitochondrion **2,** 71-86.

Jaschkowitz, K., and Seidler, A. (2000). Role of a NifS-like protein from the cyanobacterium *Synechocystis* PCC 6803 in the maturation of FeS proteins. Biochem. **39,** 3416-3423.

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

Kiley, P.J., and Beinert, H. (2003). The role of Fe-S proteins in sensing and regulation in bacteria. Curr. Opin. Microbiol. **6,** 181-185.

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.

Leibrecht, I., and Kessler, D. (1997). A novel L-cysteine/cystine C-S-lyase directing [2Fe-2S] cluster formation of *Synechocystis* ferredoxin. J. Biol. Chem. **272,** 10442-10447.

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

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

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

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

Patzer, S.I., and Hantke, K. (1999). SufS is a NifS-like protein, and SufD is necessary for stability of the [2Fe-2S] FhuF protein in *Escherichia coli*. J. Bacteriol. **181,** 3307-3309.

Pilon-Smits, E.A., 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.

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

Tong, W.H., and Rouault, T. (2000). Distinct iron-sulfur cluster assembly complexes exist in the cytosol and mitochondria of human cells. EMBO J. **19,** 5692-5700.

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.

UJWALA WAREK
P.O. Box 11141, Blacksburg, VA 24062 • *uwarek@vt.edu* • (540) 9613280

EDUCATION

- Ph.D. Molecular and Cellular Biology; Virginia Tech, Blacksburg, VA. (2003)
  Dissertation: Genes from *Arabidopsis* involved in iron-sulfur cluster biogenesis
- M.S. Microbiology, Virginia Tech, Blacksburg, VA. (1990)
  Thesis topic: Action of Clofazimine on the *Mycobacterium avium* complex
- B.S. Biology, Virginia Tech, Blacksburg, VA. (1987)
- A-Levels, Cambridge Board, Mbabane, Swaziland. (1983)

WORK EXPERIENCE

- PRODUCT MANAGEMENT – (2 years) – Technical consultant for Eudragit® and Eupergit®. Job involved international travel (50% of time) to India, South East Asia and the Far East from the German base and meeting with local agents and customers. Presenting talks and holding workshops for on-site troubleshooting. In Germany, organized training sessions for new and old customers, solved product related problems with company pharmacists.
- BUSINESS MANAGEMENT – (6 years) – established and managed a company that exported industrial equipment from Europe to Far and South East Asia and, the Middle East. Participated in Bids for supplying equipment to government agencies and private sector companies along with local agents.
- LABORATORY EXPERIENCE – (8 years)
  MOLECULAR BIOLOGY – DNA, RNA and protein isolation; Primer engineering, PCR and RT-PCR; DNA sequencing; Mutagenesis, Cloning, RNA silencing; Transformation of Bacteria and Plants; SDS-PAGE, Southern, Western and Northern Blots; Recombinant Protein purification, Protein expression assays in Plants and Bacteria, Promoter analysis, Functional genomics and proteomics; DNA Star (DNA, RNA and Protein sequence and Phylogenetic tree analysis), Workbench
  MICROSCOPY – Proficient in Confocal Laser Microscopy
- PRACTICAL TRAINING – Aspects of Sustained release formulations, Coating of Tablets, Granules and Powders. Manufacture of Tablets and Capsules. Marketing training in the USA with Roehm & Haas.
- SUPERVISORY EXPERIENCE – (11 years)
  - 2 years – As Product Manager ensured smooth running of orders. Kept abreast with manufacturing and export personnel with any complications arising due to production or delivery delays. Supervised local agents in various countries regarding samples, stocks and answering technical questions
  - 6 years – Managed a group of 5 people while working with Export Company. Ensured correct sourcing of items, pricing, and timely submission of quotations, delivery times, payment methods and schedules as well as delivery of items. Met deadlines.

    - 3 years – During Ph.D. studies, supervised the research projects of two undergraduate students. This included planning experiments with them, providing training in techniques and helping to solve technical problems.
- TEACHING EXPERIENCE – (5 years) – Independently taught lab courses (50 – 75 students per semester) in Molecular Biology, Pathogenic Bacteriology and the Principles of Biology. In addition to teaching, responsibilities included designing and administering examinations, as well as grading.
- LANGUAGES – Fluent in English, German, Hindi and Marathi
- OTHER – culturally sensitive – lived, studied and worked in various countries and cultures

BUSINESS EXPERIENCE

1998 – 2000	Manager, A2 Import Export GmbH, Hamburg, Germany. Export of Industrial Equipment to Asia, Middle and the Far East.
1994 – 1997	Manager, German Industrial Suppliers, Hamburg, Germany. Export of Industrial Equipment to Asia, Middle and the Far East.
1991 – 1993	Product Manager, Röhm GmbH, Darmstadt, Germany for the regions of India, South East Asia and the Far East. Technical consultant for the use of Methyl methacrylate polymers EUDRAGIT® used in various sustained release drug formulations, and EUPERGIT® used as matrix material in columns

PUBLICATIONS

- **Ujwala Warek** and Joseph O. Falkinham III. 1996. Action of Clofazimine on the *Mycobacterium avium* complex. Res. Microbiol. 147(1-2):43-48.
  Manuscripts in preparation
- Ana G. P. Frazzon, **Ujwala Warek**, Jeverson Frazzon, Dennis R. Dean, Brenda S. J. Winkel. AtNFS1 mediates iron-sulfur cluster assembly in *Arabidopsis* mitochondria.
- **Ujwala Warek**, Ana G. P. Frazzon, Brenda S. J. Winkel. Role of the *AtISU* genes in iron-sulfur biogenesis in *Arabidopsis*.

PRESENTATIONS AT INTERNATIONAL CONFERENCES

- Gordon Conference on Cell Macromolecular Organization and Function, Oxford, U.K. 2002
- Fe S Biogenesis, Structure, Function and Evolution. Phillips Universität, Marburg, Germany. 2002
- BioIron International Conference, Bethesda, Maryland, USA. 2003
- 14th International Conference on *Arabidopsis* Research. Univ. of Wisconsin-Madison. USA. 2003

ACADEMIC AWARDS

- Graduate Research Development Project (GRDP), Virginia Tech. 2000, 2001 and, 2003
- GRDP Travel Grant, Virginia Tech. 2002
- Virginia Academy of Sciences. 2001 and 2003
- The Society for Experimental Biology U.K. 2002
- Gordon Research, Oxford, UK Conference. 2002

PROFESSIONAL AFFILIATIONS

- International BioIron Society
- The Society for Experimental Biology, U.K.
- American Society for Plant Biology, USA
- Virginia Academy of Sciences, USA

EXTRA CURRICULAR UNIVERSITY ACTIVITIES

- Faculty Search Committee member - Fralin Biotechnology Center, (2003).
- Biotech Conference Organization, Fralin Biotechnology Center, (2003).
- Virginia Representative in Germany, HOKIES Abroad Program, (1995-2000).
- Graduate Student Representative in Office of the Provost - Curriculum Planning and Coordinating Committee, (1989-90).