Probing Plant Metabolism: 
The Machineries of [Fe-S] Cluster Assembly and Flavonoid Biosynthesis

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The organization of metabolism is an essential feature of cellular biochemistry. Metabolism does not occur as a linear progression of reactions catalyzed by freely diffusing enzymes, but as a complex web in which multiple enzyme interactions are possible. Because of the crowded environment of the cell, there must be structured and ordered mechanisms that control metabolic pathways. The following work will examine two metabolic pathways, one that is ubiquitous among living organisms and another that is entirely unique to plants, and examine the organization of each in an attempt to further define mechanisms that are fundamental features of metabolic control. These two distinct pathways serve as unique models for genetic and biochemical studies that contribute to our overall understanding of plant metabolism.

The first study offers some of the first characterizations of genes involved in iron-sulfur ([Fe-S]) cluster assembly in *Arabidopsis*. Machinery for the assembly of the [Fe-S] clusters that function as cofactors in a wide variety of proteins has been identified in microbes, insects, and animals. Homologs of the genes involved in [Fe-S] cluster biogenesis have recently been found in plants, as well, and point to the existence of two distinct systems in these organisms, one located in plastids and one in mitochondria. Here we present the first biochemical confirmation of the activity of two components of the mitochondrial machinery in *Arabidopsis*, AtNFS1 and AtISU1. Expression of the corresponding genes, as well as AtISU2 and AtISU3, and the phenotypes of plants in
which these genes are up or down-regulated were shown to be consistent with a role for
the mitochondrial [Fe-S] assembly system in the maturation of proteins required for
normal plant development.

The second study explores the mechanisms that control localization of an
enzyme that is part of the well-characterized flavonoid biosynthetic pathway. Flavonoids
are well-known plant metabolites that perform a variety of key physiological functions in
plants. Until recently, it was believed that flavonoids were synthesized exclusively in the
cytoplasm and then transported to sites of action within the cell. However, new evidence
indicates the presence of at least two flavonoid enzymes, chalcone synthase (CHS) and
chalcone isomerase (CHI), in the nucleus, suggesting that the synthesis of nuclear
flavonoids may occur in situ. CHS contains a sequence similar to a classic nuclear
localization signal (NLS), while CHI does not. The current study explores the role of
specific amino acid residues in the nuclear localization of CHS and addresses the
possibility that subcellular localization of flavonoid enzymes determines the types and
cellular locations of the end products that are produced. Confocal microscopy was used
to examine the localization of CHS expressed from the native promoter as a fusion to
green fluorescent protein in stably-transformed plants. Site-directed mutagenesis was
used to probe the role of the specific amino acid residues, including the putative NLS, in
localization of CHS. This work represents an essential step toward elucidating the
mechanisms that organize related metabolic enzymes within the cell.
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ABBREVIATIONS

AO - Aldehyde oxidase
BAC - Bacterial artificial chromosome
CAMV - Cauliflower mosaic virus
CLSM - Confocal laser scanning microscopy
EST - Expressed sequence tag
GUS - b-Glucuronidase
GFP - Green fluorescent protein
[Fe-S] - Iron–sulfur
NIF – Nitrogen fixation
ISC - Iron sulfur cluster
MpCo - Molybdenum co-factor
nos - Nopaline synthase
NPQ - Non-photochemical quenching
PSI - Photosystem I
PSII - Photosystem II
PCR - Polymerase chain reaction
PLP - Pyridoxal 5¢-phosphate
qP - Photochemical quenching
$\phi_{\text{PSI/PSII}}$ - Quantum yield
RT-PCR - Reverse transcriptase
TEV - Tobacco Etch Virus
TRX - Thioredoxin
WT - Wild type
NLS - nuclear localization signal
SPR – Surface plasmon resonance
CHS – chalcone synthase
CHI – chalcone isomerase
FLS – flavonol synthase
F3’H – flavonoid 3’ hydroxylase
DPBA – diphenylboric acid-2-aminoethyl ester
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Probing Plant Metabolism: The Machineries of [Fe-S] Cluster Assembly and Flavonoid Biosynthesis

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Chapter 1

Literature Review

Figure 1 reprinted from Biochimica Et Biophysica Acta, Vol. 1459, U. Muhlenhoff and R. Lill, “Biogenesis of iron-sulfur proteins in eukaryotes: a novel task of mitochondria that is inherited from bacteria,” pages 370-382, 2000, with permission from Elsevier.

Figure 3 reprinted from Trends in Biochemical Sciences, Vol. 25, P.A. Srere, “Macromolecular interactions: tracing the roots,” pages 150-153, 2000, with permission from Elsevier.
INTRODUCTION

The organization of metabolism is an essential feature of cellular biochemistry. Metabolism does not occur as a linear process carried by of freely diffusing enzymes, but as a complex web in which multiple interactions are possible. Because of the crowded environment of the cell, there must be structured and ordered mechanisms that control metabolic pathways. The following work will examine two metabolic pathways found in plants and examine the organization of each in an attempt to further define mechanisms that are fundamental features of metabolic control.

The first study is a functional characterization of plant genes involved in the ancient metabolic pathway of [Fe-S] cluster biogenesis. Proteins utilizing [Fe-S] clusters have roles in critical life-sustaining processes. While this metabolic pathway is well detailed in bacteria, yeast, and mammals, little is known about how this system works in plants. The current study presents the first characterization of *Arabidopsis* genes involved in mitochondrial [Fe-S] cluster assembly.

The second study is focused on the well-characterized pathway for flavonoid biosynthesis. Plants have developed an arsenal of compounds for protection against biotic stresses such as microbial infection and herbivory by insects and animals, and abiotic stresses such as UV light, temperature extremes, and drought. These specialized metabolites contribute to the overall fitness of plants and have allowed them to successfully colonize diverse ecological niches. The flavonoid pathway is also interesting from the standpoint of agricultural engineering. There has been work focused on the potential for engineering plants to overproduce specific flavonoids of economic or medicinal value (Chapple et al., 1994; Tanaka and Ohmiya, 2008). Yet much remains to be understood about the organization and regulation of the pathway before this can be done in an efficient and effective manner.
The study presented here focuses on the characterization of two distinct metabolic pathways, one that is ubiquitous among living organisms and another that is entirely unique to plants. Both pathways serve as unique models for genetic and biochemical studies that contribute to our overall understanding of plant metabolism. By examining both pathways we can make observations and conclusions about the underlying features of metabolism organization that are applicable to any metabolic pathways.
[Fe-S] CLUSTER ASSEMBLY

Introduction

Iron-sulfur [Fe-S] clusters are ubiquitous prosthetic groups found in many different types of proteins (reviewed in Johnson et al., 2005). Proteins utilizing [Fe-S] clusters are found throughout the cell and have roles in life-sustaining processes such as electron transport, nitrogen fixation, hormone biosynthesis, and environmental sensing. All organisms studied to date use some form of the [Fe-S] cluster to carry out these processes. Despite the fact that these clusters occur throughout nature, free iron and sulfur are toxic to cells, so it is likely that a regulated mechanism for their assembly also exists in all organisms. Proteins responsible for [Fe-S] cluster assembly have been identified in microbes and animals and retain a high degree of nucleotide sequence similarity between organisms. Although the importance of these proteins is illustrated through their conservation, their specific functions are only now surfacing. While some understanding has been gained from studies in prokaryotes, yeast, and humans, the precise mechanism by which these clusters are formed in vivo and the subsequent insertion into the apoprotein has remained elusive. While the proposed mechanism has been supported by several in vitro studies (Agar et al., 2000), only recently have new approaches been developed to show cluster formation in vivo (Raulfs et al., 2008). Despite these advances, there has been little focus on this assembly system in plants until recently. With the characterization of these proteins in a model system such as Arabidopsis thaliana, a greater understanding of the mechanism of [Fe-S] cluster biogenesis can be developed.
[Fe-S] cluster assembly in bacteria

The nif, isc, and suf operons

The first insights into the mechanism of [Fe-S] cluster biogenesis came from studies carried out in the nitrogen-fixing bacterium, *Azotobacter vinlandii*. With the discovery of the *nif* (nitrogen fixation) operon came the identification of numerous genes whose functions were critical for full nitrogenase activity (Jacobson et al., 1989). The nitrogenase enzyme is a complex metalloenzyme comprised of two components, the Fe protein and the MoFe protein, both of which contain [Fe-S] clusters (Zheng et al., 1993). The presence of the cluster in each protein component is required for full nitrogenase activity (Frazzon et al., 2002). This led to the identification of two key proteins, NifS and NifU, encoded by the *nifS* and *nifU* genes respectively (Zheng et al., 1993). Mutants of *A. vinlandii* that are not able to produce functional NifS or NifU are severely inhibited in their ability to produce active nitrogenase. In addition to NifS and NifU, a variety of other gene products encoded by the *nif* operon are required for full nitrogenase activity. However, when NifS and NifU are not functional, other proteins known to have [Fe-S] clusters retain their activity, indicating that another mechanism of biogenesis is present (Jacobson et al., 1989). An alternate [Fe-S] cluster assembly system, encoded by the *isc* (iron-sulfur cluster) operon, was shown to be responsible for cluster assembly in proteins other than nitrogenase (Zheng et al., 1998). Recently, a third cluster assembly operon was identified in *E. coli* through its role in sulfur trafficking in the cell (Outten et al., 2003). The *suf* operon appears to function primarily under conditions of stress. While three distinct operons appear to be responsible for alternate [Fe-S] cluster assembly systems, critical components are conserved among them, suggesting a common evolutionary origin (Muhlenhoff and Lill, 2000)(Figure 1).
Structure and function of the NifS protein and its homologs

Biochemical studies have shown NifS to be a homodimer containing a pyrodoxal-5'-phosphate (PLP) cofactor (Zheng et al., 1993). *In vitro* biochemical assays revealed the ability of NifS to bind sulfur from cysteine, converting it to alanine via the PLP cofactor (Zheng et al., 1993). Mutants of *A. vinlandii* with nonfunctional NifS, were still able to catalyze the same enzymatic reaction by using IscS in *in vitro* biochemical studies (Zheng et al., 1998). SufS, the NifS homolog of the suf operon, also exhibits cysteine desulfurase activity (Mihara et al., 1999). Most suf and isc mutants of *E. coli* are lethal, indicating the need for at least one properly-functioning set of machinery for viability (Takahashi and Tokumoto, 2002).

Figure 1: Organization of [Fe-S] cluster biogenesis gene families in bacteria and eukaryotes (Muhlenhoff and Lill, 2000). Critical genes needed for [Fe-S] biogenesis have homologs throughout nature. (reprinted with permission)
Structure and function of the NifU protein and its homologs

The NifU protein functions as a homodimer and itself contains a permanent \([2\text{Fe}-2\text{S}]^{2+}\) cluster (Yuvaniyama et al., 2000). It has also been shown that NifU can interact directly with NifS. *In vitro* results show that in the presence of NifS, ferric iron, and L-cysteine, a second, transient cluster can be formed on NifU (Yuvaniyama et al., 2000). IscU, which is homologous to the N-terminus of NifU, has been shown to have the ability to accept both \([2\text{Fe}-2\text{S}]^{2+}\) and \([4\text{Fe}-4\text{S}]^{2+}\) cluster in vitro (Agar et al., 2000). In the *suf* system, the SufA protein appears to function as the scaffold to accept sulfur from SufS (Ollagnier-de Choudens et al., 2003). However, SufA does not share homology with NifU or IscU, indicating that the *suf* system is highly specific (Ollagnier-de Choudens et al., 2003).

Mechanism of [Fe-S] assembly

The general mechanism for [Fe-S] assembly in bacteria involves both the S protein (NifS, IscS, or SufS) and the U protein (NifU, IscU, or SufA) (Figure 2) (Yuvaniyama et al., 2000; Ollagnier-de Choudens et al., 2003). The S protein functions as a cysteine desulfurase, binding a sulfur atom from a conserved cysteine residue and converting that residue to alanine. The U protein binds iron by a mechanism that is still unknown and functions as a scaffold on which the cluster is assembled. The S protein delivers the sulfur to the U protein where cluster assembly occurs. The U protein then delivers the cluster to the target apoprotein.
Previous studies focused on dissecting the mechanism of [Fe-S] cluster assembly have relied on in vitro techniques (Agar et al., 2000). Only recently have new approaches been developed to show cluster formation in vivo (2008). Raulfs et al. have reported the isolation of IscU from a modified A. vinlandii that retained a [2Fe-2S] cluster, confirming that IscU functions as a cluster scaffold. The work represents the first in vivo confirmation of previous in vitro data.

[Fe-S] cluster assembly in yeast

Studies in the yeast model organism, Saccharomyces cerevisiae, have begun to provide insights into [Fe-S] biogenesis in eukaryotes. S. cerevisiae contains homologs to most genes in the nif and isc operons (Strain et al., 1998; Kispal et al., 1999). Sequence analysis of these proteins suggested that they are targeted to mitochondria. In fact, in the eukaryotic cell, the majority of [Fe-S] cluster proteins are located in the

Figure 2: General mechanism for [Fe-S] biogenesis in bacteria. The IscS protein binds sulfur from cysteine, converting it to alanine, and delivers it to the IscU protein. The IscU protein contains three conserved cysteine residues, which function as the scaffold on which the cluster is assembled, and binds iron. The IscU protein delivers the cluster to the target apoprotein.
mitochondria (Lill and Kispal, 2000) due to the reducing environment required for cluster formation and for retaining cluster integrity. This raised the possibility that mitochondria are the primary site for [Fe-S] cluster biogenesis for the eukaryotic cell. If this is so, then a unique mechanism for their export into the cytoplasm must be present or another, distinct set of machinery is present in the cytosol (Lill et al., 1999). The former was shown to be true based on studies focused on the mitochondrial ABC transporter, Atm1p (Kispal et al., 1999). Mutants of S. cerevisiae with a deletion of the ATM1 gene showed deceased levels of cytoplasmic [Fe-S] proteins, while there was no change in the levels of mitochondrial [Fe-S] proteins. Export of the cluster is therefore dependent on a functional transporter since there is no machinery in the cytoplasm. Thus, while the overall mechanism of [Fe-S] cluster biogenesis and subsequent insertion into the apoprotein in yeast resembles the mechanism used by bacteria, further steps are needed for the successful export of clusters from mitochondria. This also demonstrates that the synthesis and export of [Fe-S] clusters is one task performed by mitochondria that make the organelle indispensable to the eukaryotic cell (Lill et al., 1999).

**[Fe-S] cluster assembly in plants**

The mechanism of [Fe-S] cluster biogenesis in plants is just beginning to be investigated. Like yeast, plants appear to contain homologs of many of the prokaryotic genes required for this process. For example, the genome of Arabidopsis contains two homologs to the bacterial nifS and iscS genes, designated AtNFS1 and AtNFS2 (Kushnir et al., 2001; Tachezy et al., 2001; Léon et al., 2002; Pilon-Smits et al., 2002; Frazzon et al., 2007). Arabidopsis also has three genes with homology to iscU and the N-terminus of nifU, designated AtISU1, AtISU2, and AtISU3 (Frazzon et al., 2007) and five genes
with homology to the C-terminus of \textit{nifU}, designated \textit{AtNUF1}, \textit{AtNUF2}, \textit{AtNUF3}, \textit{AtNUF4}, and \textit{AtNUF5} (Léon et al., 2003).

\textit{AtNFS1} is localized to mitochondria (Frazzon et al., 2007), while \textit{AtNFS2} is localized to chloroplasts (Léon et al., 2002; Pilon-Smits et al., 2002). \textit{AtNFS1} is likely critical for providing clusters to numerous proteins involved in electron transport. \textit{Arabidopsis} plants with reduced levels of \textit{AtNFS2} exhibit chlorosis and disruptions in other chloroplast specific processes, but have no effect on mitochondrial proteins utilizing [Fe-S] clusters (Van Hoewyk et al., 2007). This confirms the hypothesis that plants contain multiple machineries in distinct organelles for the production of [Fe-S] clusters.

Although the importance of the genes involved in [Fe-S] biogenesis is illustrated through their conservation across organisms, little is known about the structure and function of the gene products in plants. Plants provide a unique system for the study of [Fe-S] cluster assembly because they contain chloroplasts, a potential location for distinct [Fe-S] cluster assembly machinery. The goal of this work is to characterize the plant genes and proteins involved in mitochondrial [Fe-S] cluster biogenesis and to begin to determine the related physiological roles of these proteins.
FLAVONOID BIOSYNTHESIS

Introduction

Flavonoid biosynthesis is a well-characterized metabolic pathway that is found exclusively in plants. The products of this pathway, which are derived from phenylalanine and acetyl CoA (Stafford, 1990), comprise a diverse set of compounds that perform a variety of physiological functions in the plant cell. Flavonoids are well known for their role as the major red, blue, and purple pigments in plants that are used to attract pollinators (Weiss, 1991). These products also provide critical protection against UV radiation (Li et al., 1993), microbial infection, and herbivory (Stafford, 1990). Increasing attention has also been focused on the antioxidant and anticancer properties of flavonoids with regard to human health (Harborne and Williams, 2000).

As early as the 1940’s, the idea of multienzyme complexes began to surface as a way the cell might organize enzymes to enhance the efficiency of metabolism (reviewed in Srere, 2000). It has now become evident that the organization of macromolecular interactions is a fundamental aspect of cellular biochemistry. The flavonoid pathway provides an excellent model for studying the organization and structure of enzyme complexes because it is so amenable to genetic and biochemical characterization. While the central steps of flavonoid biosynthesis are well detailed (Winkel-Shirley, 2001), the pathway is complicated by the fact that many of the enzymes are able to utilize several different substrates and may also engage in protein-protein interactions with each other. This raises the possibility that the pathway might not exist as a linear assembly of enzymes, but as a complex web in which interactions may occur as a result of, or in response to, a variety of factors. Because of opportunities for multiple
interactions, it is unlikely that either the enzymes or substrates are found throughout the cytoplasm.

The Winkel laboratory uses the flavonoid pathway of *Arabidopsis thaliana* as a model to understand the assembly and regulation of enzyme complexes. In order to explore the idea that different flavonoids have specialized functions depending on where they are located within the cell, studies are focused on determining the subcellular localization of enzymes of the pathway, and therefore locations in which flavonoids would be expected to accumulate. Three-dimensional structures for several of these enzymes are being used to explore which structural components, including specific amino acids, affect localization, and assembly of these enzymes into complexes. As the details of the structures and functions of this pathway emerge, it should be possible to extend this knowledge to other plant systems with the goal of providing information for rational and targeted genetic engineering of agricultural crops. Moreover, it is very likely that the information will have applications to many other systems, as macromolecular organization increasingly appears to be a fundamental feature of cellular metabolism.

**The Flavonoid Biosynthetic Pathway**

*Secondary metabolism in Arabidopsis*

Because plants are quite literally rooted in place, there are limited ways in which these organisms can respond to external stresses. Thus, plants have developed an arsenal of compounds for protection against biotic stresses such as microbial infection and herbivory by insects and animals, and abiotic stresses such as UV light, temperature extremes and drought. These compounds, traditionally referred to as
secondary metabolites, have been regarded as such because they were initially not believed to be essential for growth and development. However, as *Arabidopsis* has become a popular model for studies, encompassing countless areas of plant biology, more and more is being learned about the novel roles of these compounds. In just ten years, the number of known specialized metabolites has increased five-fold and expanded into seven major classes (reviewed in D'Auria and Gershenzon, 2005). Once thought to be only of ecological importance for plant defense, these specialized metabolites are emerging as excellent candidates for molecular and genetic studies. As the diverse functions of these compounds are increasingly understood and as the biosynthetic pathways dissected, there is the potential for engineering plants to overproduce specific metabolites of economic or medicinal value (Chapple et al., 1994). However, it is also clear that much more remains to be understood about the organization and regulation of cellular metabolism before this can be done in an efficient and effective manner.

Flavonoids: structure and function

The basic flavonoid structure consists of a C-15 ring system generated through the first two steps of the pathway. From this basic structure, over 6,000 unique flavonoid compounds are known to be derived in nature, making up the six main subgroups found in higher plants: chalcones, flavones, flavonols, flavandiols, anthocyanins, and proanthocyanadins (reviewed in Winkel-Shirley, 2001).

One significant role of flavonoids is the protection they provide for plants from ultraviolet light, particularly in the UV-B range. Flavonoids, specifically the flavanol kaempferol and its derivatives, are known to accumulate in the epidermal cells in leaves and are able to efficiently absorb UV-B radiation (Li et al., 1993). Studies in *Arabidopsis*
first demonstrated that flavonoids were important for protection against UV-B irradiation by showing that flavonoid mutants that were not able to synthesize these protective compounds were more sensitive to exposure to UV-B radiation (Li et al., 1993). However, later studies have indicated that mutants deficient in sinapate esters, another commonly-found class of specialized aromatic metabolites, showed even stronger sensitivity to UV-B exposure, suggesting that plants have likely evolved several types of UV protectants (Landry et al., 1995).

The other numerous roles of flavonoids are diverse, encompassing signaling between plants and symbiotic bacteria, facilitating nitrogen fixation by the flavones apigenin and luteolin, and serving as feeding deterrents against herbivory from animals and insects by the bitter-tasting proanthocyanidins (Buchanan et al., 2000). Flavonoids also defend plants against microbial attack, as in the case of Staphylococcus infections, where the retrochalcone licochalcone C has been shown to have antimicrobial activity (reviewed in Harborne and Williams, 2000). Compounds in the subclass anthocyanins contribute to color in flowers and fruit that is important for attracting pollinators and seed dispersers (Buchanan et al., 2000). Because flavonoids, specifically anthocyanins, are responsible for providing beautifully colored flowers, there is also commercial value for these products.

Increasing attention is being focused on the possible effects of flavonoids on human health (Harborne and Williams, 2000). For example, the antiviral properties have emerged as an area of intense research, especially with regard to studies with HIV, which has been shown to exhibit some sensitivity to the flavonoid baicalin (Li et al., 2000). There have also been recent studies focused on exploring the possible benefits of plant phenolic compounds as a treatment for Alzheimer’s diseases. Moderate consumption of red wine was shown to reduce amyloid plaque formation, a hallmark of the disease, in a mouse model (Wang et al., 2006). Dietary flavonoids are thought to
serve as antioxidants, anticancer agents, and to be preventative against a number of other ailments (Yao et al., 2004).

However, it is also important to consider the negative effects that flavonoids can produce when ingested. The flavonol quercetin and its derivatives, for example, have been shown to be mutagenic and carcinogenic in bacteria and mammalian cells when provided in high doses (Rietjens et al., 2005). A recent study found a potential link between dietary flavonoids during pregnancy and autism due to the antithyroidal properties of flavonoids, likely a result of their structural similarity to thyroid hormones (Roman, 2007). Thus, the possible pharmacological applications of flavonoids to human health are an area of great interest to many disciplines, but must be approached with caution until all possible effects, both desired and unintended, have been evaluated.

*Enzyme complexes*

In the early part of the last century, the cell was viewed as simply a bag of enzymes and little attention was focused on the structural organization within (reviewed in Srere, 2000). One of the first challenges to this view came in the 1940s from David Green’s work with the tricarboxylic acid (TCA) cycle, in which he reported the isolation of aggregates containing all of the enzymes of this pathway (Green, 1958). In the 1960s, independent experiments by Zalokar (1960) and by Kempner and Miller (1968) (Figure 1) showed that ultracentrifugation techniques could be used to stratify the contents of intact cells. It was noted that few to no enzymes were present in the soluble fraction, and the conclusion was that enzymes must be associated with cellular structures. These classical experiments provided compelling evidence that the cell had a highly organized structure.
The organization of cooperating enzymes into complexes, or metabolons, can be useful to the cell in many ways. It allows for the separation of anabolic and catabolic processing of compounds (reviewed in Hrazdina and Jensen, 1992). This organization also allows the accumulation of high local substrate concentrations and the channeling of intermediates between active sites, and ensures that toxic intermediates are sequestered away from the bulk of the cell. The idea of metabolic channeling can be traced back to the 1950s when Yanofsky and Rachmeler (1958) observed that, during tryptophan biosynthesis in *Neurospora crassa*, the intermediate indole was not found in the aqueous phase of the cell. This was later confirmed by structural studies that showed that the tryptophan synthase complex from *Salmonella typhimurium* forms a tunnel in which the intermediate is channeled from one active site to the next (Hyde et al., 1988; reviewed in Dunn et al., 2008). These examples illustrate instances in which enzyme complexes are used as an efficient mechanism for regulating metabolism, providing further evidence that there is a highly organized macromolecular structure within the cell.

**Figure 3:** *Euglena* cell stratified by ultra centrifugation, from the work of Kempner and Miller (Srere, 2000). (reprinted with permission)
Flavonoid biosynthesis begins with the condensation of 4-coumaroyl-CoA with three molecules of malonyl-CoA (Figure 4). 4-coumaroyl-CoA is derived from phenylalanine that is obtained from the shikimic acid pathway and modified in a series of steps referred to as the general phenylpropanoid pathway. Malonyl-CoA, derived from acetyl-CoA, is a product of fatty acid biosynthesis. This first committed step is carried out by the homodimeric polyketide synthase, chalcone synthase (CHS), producing tetrahydroxychalcone. Chalcone isomerase (CHI) then performs the reversible isomerization of tetrahydroxychalcone to naringenin, which has the basic C-15 ring structure that all flavonoids share. At this point, the pathway begins branching to derive several classes of flavonoids. Naringenin can be converted to dihydrokaempferol by the 2-oxoglutarate dependent dioxygenase (2ODD), flavanone 3-hydroxylase (F3H). Both naringenin and dihydrokaempferol are substrates for the cytochrome P450 enzyme, flavonoid 3' hydroxylase (F3'H), forming eriodictyol and dihydroquercetin, respectively. F3H can also convert eriodictyol to dihydroquercetin. Dihydrokaempferol and dihydroquercetin can be modified by the 2ODD enzyme, flavonol synthase (FLS), for flavonol production. The short chain dehydrogenase/reductase (SDR), dihydroflavonol 4-reductase (DFR) uses dihydroquercetin to form leucocyanidin, which is the substrate for anthocyanin and proanthocyanidin production.
Figure 4: Schematic of flavonoid metabolism. Protein structures in blue represent the three enzymes in the pathway for which crystal structures have been solved: chalcone synthase (CHS) and chalcone isomerase (CHI; from *Medicago sativa*, pdb ids 1CGK and 1EYP), dihydroflavonol reductase (DFR; from grape, 3C1T) and anthocyanidin synthase (ANS; from Arabidopsis, 1GP6). Structures in orange are homology models for, flavanone 3-hydroxylase (F3H) and flavonol synthase (FLS) (Winkel lab, unpublished) and flavonoid 3’ hydroxylase (F3’H) (Schuler lab, Rupasinghe et al., 2003) Other abbreviations: anthocyanidin reductase (ANR), cinnamate 4-hydroxylase (C4H), p-coumarate:CoA ligase (4CL), leucoanthocyanidin reductase (LAR), and phenylalanine ammonia-lyase (PAL). The step shown in gray is not favored by the Arabidopsis enzyme *in vitro*. 
Previous studies of the flavonoid pathway in *Arabidopsis*

Most of the enzymes of the central flavonoid pathway in *Arabidopsis* are encoded by single-copy genes. The one exception is FLS, for which six sequences are present, although it appears that only one encodes a functional enzyme (Owens et al., 2008). Because the products of the pathway are not essential for growth and development, genetic mutations of the flavonoid enzymes are not lethal for the plant. Mutations at these genetic loci are collectively referred to as transparent testa or (tt) alleles because of altered pigmentation in the seed coat, which is an easily identified phenotype (Shirley et al., 1995). These properties have made the pathway an excellent model for genetic and biochemical characterization.

In 1974, Helen Stafford proposed that enzymes and substrates involved in flavonoid biosynthesis were not likely to be found throughout the cytosol, but organized into multienzyme complexes associated with structures in the cell (1974). The first evidence of this organization came from Hrazdina and Wagner’s work with *Fagopyrum esculentum* hypocotyls (1985). These early hypotheses and observations naturally led researchers to *Arabidopsis* as a powerful model for dissecting the organization of the pathway further. Interactions among several enzymes of the pathway were first demonstrated in the Winkel laboratory by yeast two-hybrid, affinity chromatography, and coimmunoprecipitation methods (Burbulis and Winkel-Shirley, 1999). Yeast two-hybrid results showed that CHS was able to interact with DFR, CHI was able to interact with CHS, and DFR was able to interact with CHI, but that these interactions were dependent upon the orientation of the enzymes. Recombinant TRX-CHS and TRX-CHI were also used to affinity purify other flavonoid enzymes from crude plant lysates. Western blot analysis of the affinity purified crude plant lysates confirmed that CHS and F3H interact...
with bound CHI, and that CHI and F3H can interact with bound CHS. Moreover, a polyclonal anti-CHI antibody was able to immunoprecipitate not only CHI, but CHS and F3H, from crude plant lysates, pointing to the interaction of CHI with CHS and F3H. Taken together, these results confirmed the orientation dependent interactions among several enzymes of the flavonoid pathway.

In order to further evaluate the idea that flavonoid biosynthesis is organized into metabolons in plant cells, several types of localization studies have been performed. Immunofluorescence double-labeling of CHS and CHI revealed that both enzymes co-localize at the apical end of root epidermal and root cortex cells, and this was confirmed using double-labeled immuno-electron microscopy (EM)(Saslowsky and Winkel-Shirley, 2001). Immunolocalization was also examined in the Arabidopsis flavonoid mutant, tt7(88), in which the membrane associated protein, F3’H, is not present. In this case, small amounts of CHS and CHI were detected in the elongation zone cortex cells, in contrast to the high levels observed in wild type. This finding was consistent with the lowered accumulation of flavonoids in those cells in the mutant background (Saslowsky and Winkel-Shirley, 2001). Interestingly, immunolocalization studies have recently revealed that CHS and CHI co-localize in nuclei of root tip cells and epidermal cells of leaves (Saslowsky et al., 2005). Confirmation of this localization was obtained in vivo using transgenic plants expressing mGFP:CHI and CHS:mGFP under control of the 35S promoter and analyzed by confocal laser scanning microscopy (CLSM). Although the nuclear accumulation of flavonoids has been reported in Arabidopsis (Peer et al., 2001) as well as several other plant species such as Picea abies (Hutzler et al., 1998) and Brassica napus (Kuras et al., 1999), this work provided the first evidence of the presence of flavonoid enzymes in the nucleus.
Several years ago, the crystal structures of CHS and CHI from *Medicago sativa* were solved (Ferrer et al., 1999; Jez et al., 2000). This has allowed for the predicted structure of the *Arabidopsis* CHS:CHI bienzyme complex to be determined (Figure 4) based on homology modeling, data from small angle neutron scattering (SANS) experiments, and the use of docking algorithms (Dana et al., *in revision*). Several amino acid residues are predicted to be critical for this interaction by forming three salt bridges and two hydrogen bonds (Figures 5C and 5D). CHS is the only enzyme of the pathway that contains any sequence resembling a classic nuclear localization signal (NLS) and interestingly, upon binding of CHI, this sequence would be blocked (Figure 5). It is possible that this interaction has a role in localization either by blocking the putative NLS to restrict the complex from the nucleus, or to form new signals to direct the complex to specific locations within the cell (Saslowsky et al., 2005).

The goal of the current study was to define the dual localization of CHS and to understand the underlying mechanism that control this localization. We addressed the possibility that subcellular localization of flavonoid enzymes determines the types and cellular locations of the end products that are produced. The importance of specific amino acid residues in the nuclear localization of CHS was also explored. This work represents an essential step toward elucidating the mechanisms that organize related metabolic enzymes within the cell.
Figure 5: Homology model of the CHS-CHI enzyme complex and CHS. (A) Front view with the CHS homodimer represented in purple, CHI in yellow, active site residues labeled in red and the residues involved in CoA binding labeled in blue. B) Close up view of the interaction interface, with basic side chains shown in blue, acidic side chains in red, and polar side chains in green (Dana et al., in revision). C) Homology model of CHS showing the residues corresponding to the predicted NLS (Arg\textsuperscript{71}, Lys\textsuperscript{72}, Arg\textsuperscript{73}, and His\textsuperscript{74}) are in dark blue with Arg\textsuperscript{71} shown in blue, the residues proposed to be important for the interaction with CHI are in blue, and the mutation in \textit{tt4}(UV25) (Saslowsky et al., 2000) is shown in light blue. The image was produced using Swiss-Pdb viewer, version 3.7 (70).
SUMMARY

The work presented here is focused on the characterization of two distinct metabolic pathways, one that is ubiquitous among living organisms and another that is entirely unique to plants. The assembly of [Fe-S] cluster is an ancient metabolic pathway that provides critical function to proteins that have roles in numerous processes that are essential for life. Yet surprisingly little is known about how this system works in plants. The work presented offers some of the first characterization of genes involved in [Fe-S] cluster assembly in Arabidopsis. In contrast, flavonoid biosynthesis is a well-characterized plant-specific metabolic pathway. This has allowed for targeted approaches to explore the finer details of mechanisms of regulation, localization specifically, for the organization of these metabolic enzymes. While plants utilize both pathways, studies of each can contribute to our understanding in different ways. Although one is critical for life, it is the specialized pathway that has allowed plants to so thrive in diverse ecological niches. In exploring two distinct metabolic pathways, we have used a variety of genetic and biochemical approaches to extend our general understanding of metabolic organization.
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Chapter 2

Functional analysis of Arabidopsis genes involved in mitochondrial iron-sulfur cluster assembly


My contribution to this work includes the analysis of gene expression, the analysis of AtNFS1 promoter activity, the characterization of the phenotypic effects of AtISU1 downregulation, and the identification and characterization of AtNFS1 T-DNA insertion lines (SALK_041918 and SALK_083681).

Keywords: cysteine desulfurase activity, [Fe-S] cluster assembly, intracellular localization, mitochondria, transgenic plants

Abbreviations: Aldehyde oxidase (AO), Bacterial artificial chromosome (BAC), Cauliflower mosaic virus (CaMV), Confocal laser scanning microscopy CLSM, Expressed sequence tag (EST), β-Glucuronidase (GUS), Green fluorescent protein (GFP), Iron–sulfur [Fe–S], Iron sulfur cluster (ISC), Molybdenum co-factor (MoCo), Nopaline synthase (nos), Non-photochemical quenching (NPQ), Photosystem I (PSI), Photosystem II (PSII), Polymerase chain reaction (PCR), Pyridoxal 5’-phosphate (PLP), Photochemical quenching (qP), Quantum yield ($\Phi_{PSI}$), Reverse transcriptase PCR (RT-PCR), Tobacco Etch Virus (TEV), Thioredoxin (TRX), Wild type (WT)
ABSTRACT

Machinery for the assembly of the iron-sulfur ([Fe-S]) clusters that function as cofactors in a wide variety of proteins has been identified in microbes, insects, and animals. Homologs of the genes involved in [Fe-S] cluster biogenesis have recently been found in plants, as well, and point to the existence of two distinct systems in these organisms, one located in plastids and one in mitochondria. Here we present the first biochemical confirmation of the activity of two components of the mitochondrial machinery in Arabidopsis, AtNFS1 and AtISU1. Analysis of the expression patterns of the corresponding genes, as well as AtISU2 and AtISU3, and the phenotypes of plants in which these genes are up or down-regulated are consistent with a role for the mitochondrial [Fe-S] assembly system in the maturation of proteins required for normal plant development.
Iron-sulfur [Fe-S] clusters are among the oldest and most versatile cofactors found in nature (Beinert, 2000). Cells have exploited the structural and electronic properties of these small inorganic clusters for a wide variety of critical activities including electron transfer, catalysis, substrate activation, and environmental sensing. As a result, the basic energy metabolism pathways and systems required for growth and development, the assimilation of nutrients, and the response to stress all depend on [Fe-S] proteins. Although [Fe-S] clusters can form spontaneously in vitro, the toxicity of Fe$^{2+}$ and S$^{2-}$ necessitates specialized mechanisms for cluster assembly and insertion into apoproteins within cells. It is therefore not surprising that such mechanisms have an ancient evolutionary origin and remain an essential and highly conserved feature of all living cells.

The machinery of [Fe-S] biogenesis is represented by at least three distinct, yet structurally and functionally-related systems, designated NIF, ISC, and SUF (reviewed in Balk and Lobréaux, 2005; Johnson et al., 2005; Lill and Mühlenhoff, 2005). The first of these to be described, the NIF system, in addition to performing specialized functions in nitrogen fixation, appears to be optimized for the biogenesis of [Fe-S] clusters under anoxic or microaerobic conditions. In nitrogen-fixing bacteria such as *Azotobacter vinelandii* it functions specifically in the maturation of nitrogenase. NIF-like systems are also present in a limited number of organisms that do not fix nitrogen, including *Entamoeba histolytica* and *Helicobacter pylori*, where they play a much broader role in [Fe-S] protein maturation. The NIF system consists of two key protein partners: NifS, a pyridoxal 5’-phosphate (PLP)-dependent L-cysteine desulfurase that mobilizes sulfur from L-cysteine, and NifU, a Fe-binding protein that serves as a scaffold for intermediate cluster assembly and transfer to apoproteins.
The second system, designated ISC for iron-sulfur cluster assembly, likely represents the housekeeping system for [Fe-S] protein maturation in most living cells. In eubacteria this system is composed of IscS (homologous to NifS) and IscU (homologous to the N terminus of NifU), as well as two heat-shock cognate proteins (HscA and HscB), an alternative scaffold protein (IscA), a transcriptional regulator (IscR), and ferredoxin (Fdx) (Johnson et al., 2005). Homologs of most of these proteins are present in most organisms, including cyanobacteria, fungi, insects, plants, and animals, but do not appear to occur in archaea. In eukaryotes the primary ISC machinery is located in mitochondria, although isoforms of several of the ISC proteins have been reported to accumulate in the cytoplasm and nucleus in mammals (Balk and Lobréaux, 2005; Tong and Rouault, 2006).

A third system for [Fe-S] cluster assembly, designated SUF for its role in sulfur mobilization, occurs in numerous bacteria, in archaea, and in plant chloroplasts, but is absent from fungi and metazoa. This system is composed of SufS (homologous to NifS) and SufE, which appears to interact with SufS to form a two-component cysteine desulfurase, as well as the SufBCD ATPase complex and the SufA scaffold protein (Loiseau et al., 2003; Outten et al., 2003). In organisms such as *Thermatoga maritima* it is the primary machinery for [Fe-S] cluster biosynthesis. However, it typically has a more limited role in cluster biogenesis in *E. coli* and has been proposed to be of particular importance under iron-limiting and oxidative stress conditions in this organism and in the oxygen-generating plastid (Olson et al., 2000; Nachin et al., 2001; Ali et al., 2004; Balk and Lobréaux, 2005).

In addition to the components of these three well-defined systems, proteins with homology to the C-terminal domain of NifU, known as NFU, appear to function as additional scaffolds for cluster assembly in cyanobacteria and in multiple subcellular locations in plants and animals (Nishio and Nakai, 2000; Léon et al., 2003; Rouault and...
In humans, alternative splicing and AUG utilization results in isoforms of ISCU and NIFS, respectively, that are differentially targeted to either mitochondria or to the cytoplasm and nucleus (Land and Rouault, 1988; Tong and Rouault, 2000). Five such proteins are encoded by the Arabidopsis genome, at least three of which are targeted to plastids (Touraine et al., 2004; Yabe et al., 2004). In vitro activity studies provide further evidence that these proteins function as alternative scaffolds in cluster assembly, but whether they serve as components of the ISC, SUF, or an as-yet-unidentified assembly system remains to be determined.

Interestingly, several of the NifS/IscS/SufS proteins also serve as sulfur donors in processes unrelated to [Fe-S] cluster assembly, including the biosynthesis of thionucleosides, thiamine, biotin, lipoate, and the molybdopterin cofactor (MoCo) in diverse organisms (Amrani et al., 2000; Sagi et al., 2002; Nakai et al., 2004; Heidenreich et al., 2005; Johnson et al., 2005). NifS/IscS/SufS-like proteins are also known to abstract Se from selenocysteine (Lacourciere et al., 2000; Pilon-Smits et al., 2002; Stadtman, 2004), with physiological roles in tRNA and formate dehydrogenase H maturation in bacteria and in the detoxification of selenium in plants (Mihara et al., 2002; Van Hoewyk et al., 2005). Thus this family of proteins appears to play a broad role in the mobilization of S and Se within cells.

Although the early work on [Fe-S] cluster assembly focused largely on bacterial, yeast, and mammalian systems, a detailed model of how this process may occur in plants has recently emerged (reviewed in Balk and Lobréaux, 2005; Kessler and Papenbrock, 2005; Pilon et al., 2006). In plants, the situation is complicated by the presence of an additional organelle, the plastid, which contains a number of essential [Fe-S] proteins including key components of the photosynthetic apparatus. Maturation of these proteins appears to involve a plastid-localized SUF-like assembly machinery and also several Nfu-like scaffold proteins. Maturation of all of the other [Fe-S] proteins in
the cell relies on an ISC-like system located in mitochondria, and perhaps to some degree on a cytoplasmic ISC-like system, although there is as yet no direct evidence for the latter. It has been suggested that the machinery in these two compartments is coordinated, at least in part, by the AtSufE protein, which stimulates the cysteine desulfuration activity of both AtSufS and AtNifS. However, it is not yet clear whether AtSufE is indeed targeted to both organelles (Xu and Møller, 2006; Ye et al., 2006).

The current study is focused on the mitochondrial machinery in Arabidopsis, which includes the ABC transporters, AtSTA1, 2, and 3 (now also known as AtATM3, 1, and 2); the cysteine desulfurase, AtNFS1 (also known as AtNifS1); and the predicted scaffold proteins, AtISU1, 2, and 3, all of which are targeted specifically to mitochondria (Kushnir et al., 2001; Léon et al., 2005). The Arabidopsis sta1/atm3 mutant accumulates somewhat higher levels of nonheme, nonprotein iron than wild type and exhibits phenotypes consistent with defects in phytohormone biosynthesis and DNA repair, both of which rely on [Fe-S] proteins (Kushnir et al., 2001). Similarly, AtSufE mutants exhibit abnormal seed development and arrested embryo development (Xu and Møller, 2006). In addition, AtSTA1/ATM3 and the three AtISU proteins functionally complement mutations in the corresponding yeast genes (Kushnir et al., 2001; Léon et al., 2005) and AtNFS1 has been shown to harbor cysteine desulfurase activity in vitro (Xu and Møller, 2006). Finally, there is indirect evidence from work in potato that a NFS1 homolog in the mitochondrial matrix is required for the synthesis of the catalytic [Fe-S] cluster in biotin synthase (Picciocchi et al., 2003).

Despite these advances in our understanding of the plant mitochondrial ISC system, a role for plant NFS1 in [Fe-S] cluster assembly remains to be confirmed and there is no direct biochemical evidence for the ability of any of the predicted components to mediate [Fe-S] cluster assembly (Balk and Lobréaux, 2005). The apparent instability of recombinant AtNFS1 has hampered biochemical analysis of this protein and its
interacting partners (Kessler and Papenbrock, 2005), although its *in vitro* cysteine desulfurase activity was recently demonstrated together with its ability to interact with AtSufE in two-hybrid and BiFC assays (Xu and Møller, 2006). Here we describe a method for the purification of active recombinant AtNFS1 from *E. coli* and demonstrate its ability to participate in the assembly of [Fe-S] clusters with AtISU1, as well as with the *A. vinelandii* scaffold protein, IscU. We also confirm the mitochondrial localization of AtNFS1 and AtISU1 and 2, and compare the expression patterns of the corresponding genes at various stages of plant development. Finally, we have begun exploring the physiological functions of the mitochondrial [Fe-S] cluster assembly machinery by examining the phenotypes of plants in which expression of *AtNFS1* and *AtISU1*, 2, and 3 has been reduced or abolished.
MATERIALS AND METHODS

Expression and purification of recombinant AtNFS1 and AtSU1 in E. coli

AtNFS1 (At5g65720) and AtSU1 (At4g22220) were cloned into pET32a and pET16b (Novagen), respectively, for expression in E. coli. The AtNFS1 coding region, including the stop codon, was amplified by PCR from bacterial artificial chromosome (BAC) clone F6H11 (Arabidopsis Biological Resource Center, Columbus, OH) using Pfu DNA polymerase (Stratagene). The forward and reverse primers (Table 1) added NcoI and BamHI sites for directional cloning into pET32a. The 3’ segment of the AtSU1 (At4g22220) coding region (amino acids 27-167) that has homology to A. vinelandii IscU was amplified from expressed sequence tag (EST) clone, K3F7TP (Arabidopsis Biological Resource Center), using primers (Table 1) that added NdeI and BamHI sites for directional cloning into pET16b. The integrity of the inserts was confirmed by sequencing using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).
**Table 1:** Primers used for assembling plasmid constructs

<table>
<thead>
<tr>
<th>Gene/vector</th>
<th>Direction</th>
<th>Sequence</th>
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<tr>
<td><strong>Recombinant protein expression constructs</strong></td>
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<tr>
<td><em>AtNFS1</em> into pET32a</td>
<td>Forward</td>
<td>CATGCCATGGCGTCTAAGGTAAATC</td>
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<td></td>
<td>Reverse</td>
<td>ATCGGATCCGTTGAAATCGTGGTG</td>
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<tr>
<td><em>AtISU1</em>&lt;sub&gt;27-167&lt;/sub&gt; into pET16b</td>
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<td>CATGCAATGCGAACCTACC</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>GATCGGATCCGAATCAGCCTGTG</td>
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<td><strong>GFP fusion (localization) constructs</strong></td>
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<td><em>AtNFS1</em>&lt;sub&gt;1-59&lt;/sub&gt; into pAVA393</td>
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<td>CATGCCATGGCGTCTAAGGTAAATC</td>
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<td>Reverse</td>
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<td><em>AtISU3</em> into pCAMBIA1302</td>
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<td><strong>GUS fusion (promoter activity) constructs</strong></td>
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<td>Reverse</td>
<td>CATGCCATGGCTTATAATTCAATCTTTGAATTTATTATG</td>
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<tr>
<td><em>AtISU1</em> into pBW18</td>
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<td>into pBW18</td>
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<td>AtNFS1</td>
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<td>CATGCC<strong>ATG</strong>GCGTCTAAGGTAATC</td>
</tr>
<tr>
<td>into pBLRTL2</td>
<td>Reverse</td>
<td>CATGCCATGG<strong>TCAG</strong>GTGAGACCATTTG</td>
</tr>
<tr>
<td>AtISU1</td>
<td>Forward</td>
<td>CATGCC<strong>ATG</strong>GCCTGTGTGGTTTCTCC</td>
</tr>
<tr>
<td>into pBLRTL2</td>
<td>Reverse</td>
<td>CATGCCATGG<strong>TCAG</strong>CTGCTGTTGTTTTC</td>
</tr>
<tr>
<td>AtISU1 into pHANNIBAL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CCGCTCGAGGTGCTGCAGCAGCAGG</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>CCGGAATTCCATAGGTGTATGTATCATCC</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CCCAAGCTTATAGGTGTATGTATCATCC</td>
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</tr>
<tr>
<td>Reverse</td>
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<tr>
<td>AtISU2 into pHANNIBAL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CCGCTCGAGCTGCTGCTGCAGAAGAAACC</td>
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</tr>
<tr>
<td>Reverse</td>
<td>CCGGAATTCCGAAAAACTAAAACAACCAAC</td>
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</tr>
<tr>
<td>Forward</td>
<td>CCCAAGCTTAAAATAAAAACCAACCAAC</td>
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<tr>
<td>Reverse</td>
<td>CGCGGATCCGCTGCTGCTGCAGAAGAACC</td>
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<tr>
<td>AtISU3 into pHANNIBAL</td>
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<tr>
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<td>Reverse</td>
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</tr>
<tr>
<td>Forward</td>
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<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>CGCGGATCCCTCGATCCACTTATTTACAC</td>
<td></td>
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</tbody>
</table>

1 Sequences are written in the 5’ to 3’ direction; gene sequences are underlined, start and stop codons are in bold.

The pET32a-AtNFS1 construct was used to transform 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°C. 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 construct, pET16b-AtISU1, was transformed into *E.coli* BL21(DE3)pLys cells (Novagen). The cells were grown at 37°C in 500 ml LB-amp<sub>100 cm<sup>3</sup></sub>. 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 AtISU1, 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 AtISU1 that was estimated to be 90% pure, based on analysis on a Coomassie-stained SDS polyacrylamide gel. The native IscU protein was purified from \textit{A. vinelandii} as described by Agar \textit{et al.} (2000).

\textit{Activity assays}

Cysteine desulfurase activity was assayed as described in Zheng \textit{et al.} (1993) using 40 µg of purified recombinant protein in a 1 ml reaction. \textit{In vitro} assembly of Fe-S clusters was carried out essentially as described by Yuvaniyama \textit{et al.} (2000). The reactions were performed under anaerobic conditions in a quartz cuvette and contained 28 µM AtISU1, 0.8 µM AtNFS1, 2.5 mM β-mercaptoethanol, 0.1 mM ferric ammonium citrate, 2 mM L-cysteine, and 20 mM TrisHCl 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 \textit{A. vinelandii} and in the absence of L-cysteine, respectively. In-gel aldehyde oxidase activities were assayed as described in Bittner \textit{et al.} (2001).
**Intracellular localization**

The full-length *AtNFS1* and *AtSU1* coding regions, minus the stop codons, were amplified from clones F6H11 and K3F7TP, respectively. Reactions were carried out using *Pfu* polymerase and primers that added *NcoI* sites at both ends (Table 1). The resulting fragments were cloned in-frame with the amino terminus of mGFP5 in pAVA393 (von Arnim et al., 1998) and then excised, together with the double-enhanced cauliflower mosaic virus (CaMV) 35S promoter, Tobacco Etch Virus (TEV) translational leader, and nopaline synthase (nos) terminator sequences, with *SalI* and *XmaI* 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 CLSM following particle bombardment into onion epidermal cells (Scott et al., 1999). For *AtSU2* and *AtSU3*, the coding regions, including the introns but without the stop codons, were amplified from genomic DNA prepared as described in Watson and Thompson (1986), again using *Pfu* and primers that added *NcoI* sites (Table 1). The resulting fragments were cloned in-frame with the N terminus of mGFP5 in pCAMBIA1302 (Cambia, Canberra, Australia).

Following transformation into *Arabidopsis*, 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 CLSM analysis. Plants transformed with the empty pCAMBI1302 vector, expressing mGFP5 alone, were used as a control; these plants exhibited GFP fluorescence exclusively in the cytoplasm. Immunoblot analysis of AtSU3-GFP plants was performed as previously described (Pelletier et al., 1999) using a 1:2000 dilution of rabbit anti-GFP antibody (Clontech) and a 1:75,000 dilution of a peroxidase-conjugated goat-anti-rabbit secondary antibody (Jackson
ImmunoResearch Laboratories) followed by detection with the SuperSignal West Dura system (Pierce).

**Analysis of gene expression**

Promoter-GUS constructs were assembled by amplifying sequences preceding the start codon (from the start or stop codon of the upstream gene or ~1kb to the start codon of *AtNFS1* and *AtISU1*, 2, and 3 (908, 1410, 320, and 950 bp, respectively) from genomic DNA using primers that introduced *Nco*I sites at both ends (Table 2). The resulting fragments were cloned in front of the GUS reporter gene in mpBI121 (Zhao et al., 2005) in the case of *AtNFS1* and in the binary vector, pBW18 (a derivative of pCAMBIA1391Z in which the hygromycin resistance gene was replaced with the Bar gene; Woffenden, Jelesko, and McDowell, unpublished), for *AtISU1*, 2 and 3. Plants were stained for GUS activity as described by Sieburth and Meyerowitz (1997).

Levels of endogenous *AtNFS1* transcripts were analyzed by semi-quantitative RT-PCR. RNA was extracted from the above-ground parts of 2 month old plants as described previously (Simon et al., 1992) and treated with DNase using the TURBO DNA-free kit (Ambion). Synthesis of cDNA was carried our using the Omniscript Reverse Transcription kit (Qiagen) with an oligo(dT) primer in the presence of RNaseOut (Invitrogen). Primers (Table 3) were designed using the Primer Express software (ABI 7700: Primer Express 1.5: Primer Express™ 1.5) and purchased from Invitrogen. A plasmid containing an *AtNFS1* cDNA, pUNI51 U10851 (Arabidopsis Biological Resource Center), was used as a positive control. Amplification was for 35 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec. The expected sizes of the products were 110 bp for *AtACT2* and 1072 bp for *AtNFS1*. 
Table 2: Primers used for identification of homozygous T-DNA insertion lines by PCR and gene expression by RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Identification of homozygous T-DNA insertion lines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtNFS1</td>
<td>Forward</td>
<td>CAAGACTCTACAGACTTC</td>
</tr>
<tr>
<td>SALK_041918.39.50.x and SALK_083681.30.90.x</td>
<td>Reverse</td>
<td>GCGGGCTCATTCCCTCAAC</td>
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<tr>
<td><strong>Semi-quantitative RT-PCR</strong></td>
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<td></td>
</tr>
<tr>
<td>AtNFS1</td>
<td>Forward</td>
<td>TCGCGAAACTGATCGAAGCT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CGCACAGTTGAGTGGAACCTG</td>
</tr>
<tr>
<td>AtACT2</td>
<td>Forward</td>
<td>GCTTTTTAAGCCTTTGATCTTGAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCGGTGGTCATTCTTGCT</td>
</tr>
<tr>
<td><strong>Quantitative RT-PCR</strong></td>
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<tr>
<td>AtNFS1</td>
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<td>CGGTGCAGCTTTACGGTTAAACAGT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCGGGCTCATTCCCTCAAC</td>
</tr>
<tr>
<td>AtISU1</td>
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</tr>
<tr>
<td></td>
<td>Reverse</td>
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<tr>
<td>AtISU2</td>
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<td>AAGGCAACGTCGAGAAAGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCTAGAGGAGACGCTGTAACC</td>
</tr>
<tr>
<td>AtISU3</td>
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<td>CACACCGTTCCCTGTCGTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AATGGTCGATGACATTGGATG</td>
</tr>
</tbody>
</table>

The levels of combined endogenous and antisense AtNFS1 transcripts and endogenous AtISU1, 2, and 3 transcripts were analyzed in wild-type and transgenic plants by quantitative RT-PCR using SYBR® Green I chemistry (Applied Biosystems)
and the primers listed in Table 2. cDNA synthesis and primer design were as described for the semi-quantitative RT-PCR experiments. The reactions were incubated for 2 min at 50ºC and 10 min at 95ºC, then for forty cycles of 15 sec at 95ºC and 1 min at 60ºC, then for 15 sec at 95ºC, 20 sec at 60ºC, a 20 min ramp time up to 95ºC, and then held at 95ºC for 15 sec. Control cDNA constructs were pUNI51 U12070 (AtISU1), pENTR221-At3g01020 (AtISU2), and pENTR221-At4g04080 (AtISU3) (Arabidopsis Biological Resource Center, Columbus, OH). The experiment was performed and analyzed using an ABI 7700 instrument.

**Up and down regulation of gene expression in transgenic plants**

T-DNA insertional mutant lines for AtNFS1 (SALK_041918.39.50.x and SALK_083681.30.90.x) and AtISU1 (SALK_006332) were obtained from the Arabidopsis Biological Resource Center. Efforts to identify homozygotes for these lines involved extracting genomic DNA from wild type and segregating mutant lines using a miniprep procedure (Edwards et al., 1991), followed by PCR amplification using gene-specific primers (Table 3) with or without the left-border primer primer, Lba1 (Alonso et al., 2003). Sequence analysis of the products of two of these reactions was used to precisely define the site of this insertion. Note that the original 083681 line has now been replaced by a homozygous derivative, SALK_083681C in the ABRC collection; the other two lines are still only available as heterozygotes.

To generate sense and antisense constructs for AtNFS1 and AtISU1, a cassette containing the double-enhanced CaMV 35S promoter, TEV translational leader, and nos terminator sequences was excised from pRTL2 with HindIII and inserted into pBluescript KSP (Stratagene) to acquire polylinker sequences that facilitated subsequent cloning steps; this vector was named pBLRTL2. The AtNFS1 coding region, including the stop
codon, was amplified from F6H11; *AtISU1* sequences including the stop codon were amplified from K3F7TP. Reactions were performed using *Pfu* and primers that added *Nco*I sites at both ends (Table 2). The resulting fragments were cloned into the *Nco*I site in pBLRTL2 and insert orientation was determined by PCR and restriction digestion. The constructs, including the promoter and terminator sequences, were then excised with *Sal*I and *Kpn*I and cloned into the corresponding sites in the binary vector, pBIB (Becker, 1990).

For gene-specific down regulation of *AtISU1*, 2 and 3, RNAi constructs were generated by amplifying 120 bp from the 3’ end of each gene (83-85 bp into the UTR) with the primers shown in Table 2. These fragments were cloned in the sense and antisense orientations in the pHANNIBAL cloning vector (CSIRO, Australia and Wesley et al., 2001). For the pHANNIBAL empty vector control and *AtISU3*, a cassette that included the double enhanced CaMV promoter and nos terminator was subcloned into the binary vector, pCAMBIA3300 (Cambia, Canberra, Australia), using *Sac*I and *Pst*I. *AtISU1* and *AtISU2* RNAi cassettes were subcloned into the *Sma*I site of pCAMBIA3300 using *Not*I.

**Plant transformation and growth conditions**

The freeze-thaw method (Chen et al., 1994) was used to transform *Agrobacterium tumefaciens* strain GV3101 with the various binary constructs. *Arabidopsis* ecotype Columbia plants were transformed using the floral dip method (Clough and Bent, 1998). T1 seeds were vernalized in darkness at 4°C (on plates or soil) for 2 d and then transferred to constant white light at 22°C. For antibiotic selection, seedlings that were green after 10 d of growth on MS-sucrose plates supplemented with 100 µg/ml kanamycin or 100 µg/ml hygromycin were transferred to soil and grown
further at 22°C under a 16 h/8 h light/dark cycle. To select for herbicide resistance, seedlings growing in soil were sprayed with glufosinate ammonium (phosphinothricin, Basta®/Liberty) herbicide (AgrEvo Company, Wilmington, DE, USA), diluted 1:10000 dilution and containing 0.005% Silwet L-77 (Lehle Seeds, Round Rock, TX) starting at approximately 10 d of age for 3 d in succession with a 4 d gap (a total of 6 d of spraying); strong survivors were transplanted to fresh soil for propagation. Transgenic lines were confirmed and homozygotes were identified at the T2, T3, and T4 generations by growth on MS sucrose plates containing the selective antibiotic or by spraying with glufosinate.

For localization and promoter expression studies as well as phenotypic analysis, plants were grown in soil at 22°C under a 16 h/8 h light/dark cycle using light levels of approximately 120 µmol m⁻² s⁻¹.

**Microscopy**

Fully expanded rosette leaves from 3-4 week old plants were mounted in water under cover slips. Specimens were examined with a 40X C-Apochromat objective lens (water corrected, numerical aperture 1.2) using a LSM 510 (Carl Zeiss Inc., Thornwood, NY). Fluorescent images were obtained 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 and 650 nm long pass filters for GFP and chlorophyll, respectively. Differential interference contrast reference images were also collected. Images were exported as JPEG files and further processed with Adobe Photoshop v7.0 (Adobe Systems, San Jose, CA). Wild type plants grown under the same conditions were used as controls to account for endogenous/background fluorescence during CLSM analysis. Plants transformed with the unmodified
pCambia1302 vector were used as a control for GFP localization in the absence of a fusion partner.

**Measurement of photosynthetic capacity**

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 $\mu$mol m$^{-2}$s$^{-1}$) to determine maximal fluorescence ($F_m$) and the $F_o/F_m = (F_m - F_s)/F_m$ ratio was calculated. Leaves were then illuminated with actinic light (100 $\mu$mol 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 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.
RESULTS AND DISCUSSION

**Biochemical characterization of AtNFS1 and AtISU1**

In order to assess the ability of *AtNFS1* and *AtISU1* to carry out cluster assembly *in vitro*, the *AtNFS1* coding region was expressed in *E. coli* as a thioredoxin fusion protein (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. However, this protein was found primarily in the insoluble fraction. 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 8 M 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 1A). 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 a highly 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. Xu and Møller (2006) have reported approximately 3 nmol of sulfide min$^{-1}$ (mg of pure protein)$^{-1}$ for AtNFS1 when assayed in the absence of AtSufE; this slightly lower value may reflect differences in the expression and purification methods that were used. The activities of the *Arabidopsis* enzymes are comparable to what has been reported for *A. vinelandii* NifS (89.4 nmol of sulfide min$^{-1}$) (Zheng et al., 1993), *E. coli* NifS (78 nmol of sulfide min$^{-1}$) (Flint, 1996), *A. vinelandii* IscS (67.6 nmol of sulfide min$^{-1}$) (Zheng et al., 1998), and *Helicobacter pylori* NifS (9.96 nmol of sulfide min$^{-1}$) (Olson et al., 2000).
The ability of the recombinant AtNFS1 protein to mediate \textit{in vitro} assembly of [Fe-S] clusters was tested using the method of Yuvaniyama \textit{et al.} (2000). Reactions were performed using 0.8 µM TRX-AtNFS1 in conjunction with 28 µM of IscU purified from \textit{A. vinelandii} or recombinant AtISU1 produced in \textit{E. coli}. This experiment demonstrated the ability of TRX-AtNFS1 to mediate the time-dependent assembly of a chromophoric species in IscU and AtISU1 that exhibits the characteristic absorbance inflections of [2Fe-2S] clusters at 325, 420 and 460 nm (Figure 1B). Significantly higher activity was observed at pH 8.5 than at pH 7.4, the pH optimum for \textit{in vitro} cluster assembly by bacterial NifS/IscS proteins (data not shown).

\textit{Subcellular localization}

It has previously been shown that the predicted N-terminal targeting sequences of AtNFS1 and AtISU1, 2, and 3 direct green fluorescent protein (GFP) to mitochondria in tobacco cells (Kushnir \textit{et al.}, 2001; Léon \textit{et al.}, 2005). To confirm this localization in a native background, the full coding region for each of these proteins was fused to GFP and examined in stably transformed \textit{Arabidopsis} plants by confocal laser scanning microscopy (CLSM) (Figure 2). AtNFS1-GFP, AtISU1-GFP, and AtISU2-GFP all localized to mitochondria in multiple independent lines; no fluorescence could be detected in any other cellular location. It should be noted that none of the transformants exhibited any notable phenotypes relative to wild type. Interestingly, it was not possible to recover \textit{Arabidopsis} plants expressing detectable levels of AtISU3-GFP fluorescence from among more than 50 independent transgenic lines, even though expression was driven by the double-enhanced 35S promoter as in the other three constructs. These plants also showed no detectable levels of AtISU3-GFP protein on immunoblots analyzed with an anti-GFP antibody (data not shown). This suggests that the mRNA
and/or protein produced from AtISU3 may be substantially less stable than for the other three genes.

**Figure 2:** Localization of AtNFS1, AtISU1, and AtISU2 in Arabidopsis leaves. The ATNFS1, AtISU1, and AtISU2 coding regions were fused in-frame to the amino terminus of the plant-optimized reporter gene, mGFP5, and expressed in transgenic Arabidopsis plants under control of the constitutive double-enhanced CaMV 35S promoter. Leaves from 4-week-old plants were examined by confocal laser scanning microscopy; close-ups of stomata are shown. GFP fluorescence is shown in green; autoflorescence from chloroplasts is in red. Size bar in all panels is 5 µm.

**Phenotypic and biochemical effects of altered AtNFS1 expression**

In an effort to correlate AtNFS1 activity with specific physiological processes, mutants with altered expression levels of AtNFS1 were obtained. The SALK_041918 line (Alonso et al., 2003) has a T-DNA insertion in the coding region. Remarkably, it has not been possible to isolate a homozygous knockout for this line, suggesting that, analogous to the situation for many of the nifS- and iscS-like genes in other organisms, AtNFS1 is essential in Arabidopsis. We therefore generated transgenic plants in which the AtNFS1 coding region was placed in the reverse (antisense) orientation behind the
double-enhanced 35S promoter. Multiple independent T1 plants were recovered, of which three were isolated as homozygotes in the T3 generation. These plants exhibited a number of striking visible phenotypes. Several lines developed chlorotic spots on the leaves, as shown in Figure 3A, B, F, G, and H. 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 (Figure 3A, B, C, E, and H). These plants were also shorter than the wild-type plants at the same stage of development. Similar phenotypes, particularly the disorganized inflorescences and chlorotic spots, were also exhibited by a line carrying a T-DNA insertion in this gene, SALK_083681 (Alonso et al., 2003) (not shown). Plants homozygous for this insertion, located 149 bp upstream of the ATG and 53 bp before the predicted transcription start site based on sequence analysis of genomic PCR products, appear to be leaky with respect to mRNA accumulation as assessed by semi-quantitative RT-PCR (not shown).

Figure 3: Functional analysis of AtNFS1 by downregulation in transgenic plants. Phenotypes of plants expressing an antisense construct for AtNFS1 (panels A-C, E-I) and wild-type plants (panel D) are shown. The arrows in panels A, B, and G point to chlorotic spots on the leaves.
Defects in mitochondrial respiration, a likely consequence of decreased AtNFS1 levels, are known to affect photosynthesis, often visible as white (chlorotic) leaf sectors (Raghavendra and Padmasree, 2003). The bushy phenotype may indicate a defect in plant hormone biosynthesis, however, this pathway has not been linked to mitochondria before. Recently, it was shown that the first step of MoCo biosynthesis requires an Fe-S protein that is likely targeted to mitochondria, known as Cnx2 in Arabidopsis or MOCS1A in humans (reviewed by Mendel and Bittner, 2006). In Arabidopsis, the cytosolic MoCo-dependent aldehyde oxidase AAO3 catalyses the last step in abscisic acid biosynthesis (Seo et al., 2004). A related enzyme is also involved in one of the pathways of auxin biosynthesis. In addition to sulfurated MoCo, aldehyde oxidases (AOs) contain two [2Fe-2S] clusters. Therefore, AO activity is directly and indirectly (through MoCo biosynthesis) dependent on the mitochondrial Fe-S cluster assembly machinery. We therefore analysed the activity of AOs in anti-sense NFS1 plants using an in-gel activity assay (Bittner et al., 2001). As this assay is qualitative rather than quantitative, we sought to maximize the difference by comparing healthy looking green tissue with chlorotic tissue. We observed that the activities of all three detectable AOs were lower in the chlorotic tissue of two independent anti-sense lines (Figure 4), indicating that AtNFS1 is required for co-factor assembly on AO, affecting plant hormone biosynthesis as a consequence. It is of relevance to note that AO activities are not generally decreased in chlorotic tissues. For example, iron starved Arabidopsis seedlings or CpNifS knock-down lines displayed normal AO activities (J. Balk, manuscript in preparation).
Because Fe-S proteins play a key role in photosynthetic electron transport in the chloroplast (Imsande, 1999; Balk and Lobréaux, 2005), the AtNFS1 antisense lines were further 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 lines and 5 to 8 plants of the wild type under white light, where photosystem II (PSII) is more important, and red light, where PSI makes the greater contribution. 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 1). 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 (qP), an indication of the
proportion of PSII reaction centers that are open; and non-photochemical quenching (NPQ), which is linearly related to heat dissipation. Little or no effect on PSI function was observed in any of these lines (Table 3). There was also no measurable effect on photochemical quenching for PSII. However, all three lines exhibited a marked decrease in non-photochemical 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 may be higher than for wild type due to a decrease in the dissipation of energy as heat. Because this was the only observed effect of AtNFS1 antisense expression on PSI or PSII function in these plants, it may reflect a compensation for alterations in mitochondrial processes such as respiration, which also relies on a number of [Fe-S] proteins. Overall, the results of these experiments indicate that AtNFS1 does not participate in [Fe-S] cluster assembly in the chloroplast.
Table 3: Photosynthetic efficiency of PSII in AtNFS1 sense and antisense plants

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

a Fv/Fm, quantum yield ($\phi_{PSII}$), photosynthetic quenching (qP) and non-photochemical quenching (NPQ) were determined from chlorophyll fluorescence measurements made on plants grown 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 (numbers shown in parentheses indicate the number of plants tested for each line), together with standard error. Asterisks indicate values that differ from the wild type control with a P value <0.05.

Phenotypic effects of altered AtISU1, AtISU2, and AtISU3 expression in transgenic plants

To explore the physiological functions of AtISU1, antisense constructs were generated using a full length AtISU1 cDNA placed in reverse orientation behind the double-enhanced 35S promoter. Five independent T1 plants were identified and four homozygous lines were recovered at the T4 generation. Very similar results were
observed as for the *AtNFS1* anti-sense plants, particularly the bushy phenotype due to an increase in axillary shoot development (Figure 5A-C).

However, these phenotypes may not have been specific to *AtISU1* due to the high sequence similarity with *AtISU2* and 3. Gene-specific RNAi constructs were therefore designed using 120 bp fragments starting near the 3’ end of the coding region and extending into the 3’ untranslated region of each gene. These fragments, which showed 24-33% identity at the nucleotide level, were cloned into the pHANNIBAL vector (Wesley et al., 2001), transferred to the binary vector, and introduced into *Arabidopsis* plants. Ten to 20 independent T1 lines were recovered and analyzed for phenotypic differences for each of the constructs as well as an empty vector control.

T1 plants transformed with either the *AtISU1* or *AtISU2* RNAi had thinner stems and were generally smaller than wild-type plants (Figure 5D-G). These plants also developed numerous axillary stems, resulting in a bushy appearance, as observed for the *AtNFS1* and *AtISU1* antisense plants. Similar phenotypes were observed for the *AtISU1* T-DNA insertional mutant, SALK_006332 (Alonso et al., 2003)(not shown). Transgenic plants containing the *AtISU3* RNAi construct showed two distinct sets of phenotypes. One group of plants was similar to the *AtISU1* and *AtISU2* RNAi plants, with thinner stems and an overall smaller size, but also a slow growth rate, very early onset of bolting, and an altered rosette pattern relative to wild type (Figure 5H, right, and J). The second group exhibited an accelerated growth rate, disrupted rosette formation, and altered leaf shape relative to wild type (Figure 5H, center). These plants also produced large cauline leaves at the base of the flower. However, none of the *AtISU* antisense, RNAi, or T-DNA lines showed the chlorotic spots or the serrated leaf phenotype observed in the *AtNFS1* antisense lines, suggesting that these or other scaffold proteins have at least some redundant function. Plants transformed with an empty vector control
showed no unusual phenotypes, indicating that the observed differences from wild type were due to the presence of the transgenes.

To confirm that the phenotypes were indeed correlated with a decrease in expression of the target genes, reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was carried out on mature plants. Quantitative real-time RT-PCR confirmed that \textit{AtNFS1} mRNA levels were reduced in the antisense lines compared to wild type (Figure 6A). It was not possible to distinguish endogenous \textit{AtNFS1} transcripts from the antisense transcripts using this method due to limitations of primer design. However, endogenous \textit{AtNFS1} mRNA transcripts were undetectable in the antisense lines using a semi-quantitative RT-PCR approach (Figure 6B), indicating that there was little or no \textit{AtNFS1} expression in these plants. Quantitative real time RT-PCR revealed

\textbf{Figure 5:} Phenotypes of plants expressing an antisense construct for \textit{AtISU1} (A-C) or RNAi construct \textit{AtISU1} (D, E), \textit{AtISU2} (F, G) or \textit{AtISU3} (H center and right, J) RNAi versus phenotypes of wild-type plants (H left, I).
that endogenous mRNA levels were also substantially reduced in the *AtISU1*, 2, and 3 RNAi lines (Figure 6C).

Surprisingly, the levels of endogenous *AtSU2* and *AtSU3* mRNA were extremely low in two-month-old wild-type plants relative to *AtNFS1* and *AtSU1* (Figure 6). This difference was also apparent in the data from 2317 publicly-available chips analyzed using GeneVestigator (Zimmermann et al., 2004), which indicate that *AtNFS1* and *AtSU1* transcripts are constitutively expressed at relatively constant levels, with little or no detectable expression of *AtSU2* and *AtSU3* in most cases. Interestingly,
expression of all three *AtISU* genes appears to be induced in flowers, including pollen (Honys and Twell, 2003; Schmid et al., 2005). *AtISU1* mRNAs also accumulate to higher levels in response to far-red light and treatment with abscisic acid or gibberellin and in a catalase-deficient line (Schmid et al., 2005; Vanderauwera et al., 2005). These findings suggest that the similar phenotypes observed for the *AtISU1, 2, and 3* RNAi lines may be due to the requirement for each of these genes in very specific cells or tissues over in the course of development, perhaps below the level of detection in RNA samples from whole plants.

**Characterization of *AtNFS1, AtISU1, AtISU2, and AtISU3* promoter activity**

To examine the expression of these genes at higher resolution with regard to cell or tissue type, the 5' upstream regions, including the UTR, of the *AtNFS1* and *AtISU1, 2* and 3 genes were fused in-frame with the start codon of the GUS reporter gene in pBW18 or mpBI121. These regions were defined by the presence of the closest upstream gene, 908 bp in the case of *AtNFS1*, 1410 for *AtISU1*, and 320 for *AtISU2*. For *AtISU3*, the closest upstream annotated gene was 8.7 kb away, so 968 bp of sequence were used in this case. Multiple independent transformants were recovered for each of the constructs, of which at least ten were selected for further characterization.

Analysis of these lines indicated that both the *AtISU1* and *AtISU3* promoters were active in a wide variety of cell and tissue types at the seedling, bolting, and flowering stages of development; results for representative lines at the T2 generation are shown in Figure 7. The constructs were virtually indistinguishable with respect to both patterns and levels of expression. In contrast, no GUS activity was detected in ten independent lines for the *AtNFS1p::GUS* construct (five constructs in pBW18 and five in
mpBI121; data not shown), or in more than 30 independent lines for the *AtISU2p::GUS* construct (all in pBW18). These results are consistent with the high steady state mRNA levels for *AtISU1* and low levels for *AtISU2* observed in the qRT-PCR experiments (Figure 6A) and in the microarray databases. However, the unexpected low activity for *AtNFS1* and high activity for *AtISU3* suggest the possibility that additional regulatory sequences exist outside of the regions examined in this experiment that enhance transcription of the former and repress transcription of the latter. It is also possible that the *AtISU3* transcripts are rapidly turned over, which is consistent with the inability to recover plants with detectable levels of AtISU3-GFP protein.

![Figure 7: AtISU1 and AtISU3 promoter activity. Upstream sequences for these genes were fused to GUS and expressed in transgenic Arabidopsis plants. Histochemical staining was performed on whole plants at various stages of development.](image-url)
CONCLUSIONS

Biochemical experiments described here have provided the first direct evidence that a cysteine desulfurase and predicted scaffold protein in plants can participate in the assembly of [Fe-S] clusters. The ability of AtNFS1 to also assemble clusters in cooperation with A. vinelandii IscU indicates that the physical and functional interactions of these proteins have been conserved over an enormous evolutionary distance, the only substantial change being an apparent shift in the pH optimum for this process. Moreover, we have shown that AtNFS1 is required for the activity of aldehyde oxidases, enzymes that depend on two [2Fe-2S] clusters, FAD and MoCo. Biosynthesis of the latter co-factor involves the Fe-S enzyme Cnx2, thought to be located in the mitochondria. The presence of the mitochondrial transporter STA1/ATM3 in Arabidopsis (Kushnir et al., 2001), and work in yeast (Lill & Mühlenhoff, 2005), suggest that the mitochondrial AtNFS1 is required for the assembly of Fe-S clusters of the cytosolic Fe-S enzymes. Therefore, the activity of the cytosolic AOs may be doubly compromised by the lack of Fe-S clusters and MoCo. This remains to be further investigated. The localization of the AtNFS1 and AtISU1 and 2 proteins exclusively to mitochondria was confirmed in a native background using the full-length proteins. Phenotypes of plants in which these genes were down regulated indicate that AtNFS1 and all three AtIscU proteins contribute to the maturation of [Fe-S] proteins essential for normal plant growth and development. This is not surprising, since the mitochondrial Fe-S cluster assembly machinery is required for Fe-S clusters in the respiratory complexes and co-factor biosynthesis including biotin, lipoate and MoCo. In addition, a direct link between AtNFS1 and phytohormone biosynthesis was shown through the strongly diminished activities of AOs in anti-sense AtNFS1 lines. These enzymes are involved in the
biosynthesis of abscisic acid and auxin, two plant hormones that mediate various developmental processes, including shoot branching.

The cysteine desulphurase AtNFS1 and the scaffolds AtISU1, 2 and 3 are only part of the mitochondrial Fe-S cluster assembly pathway. It will be interesting to investigate the physiological and biochemical role of other mitochondrial components, including the NFU proteins, mitochondrial SUFE, chaperones and the transporter STA1/ATM3 and homologs. In addition, the cross talk between the mitochondrial and plastid Fe-S assembly machineries is virtually unexplored and remains an exciting field of research.
ACKNOWLEDGMENTS

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Chapter 3

Intracellular localization of chalcone synthase and effects on flavonoid accumulation

Abbreviations: Cauliflower mosaic virus (CaMV), confocal laser scanning microscopy (CLSM), β-Glucuronidase (GUS), modified green fluorescent protein (mGFP), Polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), nuclear localization signal (NLS), thioredoxin (TRX), wild type (WT)
ABSTRACT

Flavonoids are well-known plant metabolites that perform a variety of key physiological functions in plants. Until recently, it was believed that flavonoids were synthesized exclusively in the cytoplasm and then transported to sites of action within the cell. However, new evidence indicates the presence of at least two flavonoid enzymes, chalcone synthase (CHS) and chalcone isomerase (CHI), in the nucleus, suggesting that the synthesis of nuclear flavonoids may occur in situ. CHS contains a sequence similar to a classic nuclear localization signal (NLS), while CHI does not. The current study explores the role of specific amino acid residues in the nuclear localization of CHS and addresses the possibility that subcellular localization of flavonoid enzymes determines the types and cellular locations of the end products that are produced. Confocal microscopy was used to examine the localization of CHS expressed from the native promoter as a fusion to green fluorescent protein in stably-transformed plants. Site-directed mutagenesis was used to probe the role of the specific amino acid residues, including the putative NLS, in localization of CHS. This work represents an essential step toward elucidating the mechanisms that organize related metabolic enzymes within the cell.
INTRODUCTION

As early as the 1940’s, the idea of multienzyme complexes began to surface as a way the cell might organize enzymes to enhance the efficiency of metabolism (2000). Several examples now exist that illustrate how macromolecular interaction and organization are fundamental aspects of cellular metabolism (reviewed in Winkel, 2004). Modulating localization of biosynthetic enzymes could provide yet another layer of regulation. Despite its widespread occurrence, surprisingly little is known about the mechanisms that control this organization.

The flavonoid pathway of Arabidopsis thaliana offers an excellent model to understand the assembly and regulation of multienzyme complexes. Flavonoid biosynthesis is a well-characterized specialized metabolic pathway that is found exclusively in plants. All but one of the enzymes of the central flavonoid pathway in Arabidopsis are encoded by single-copy genes. The one exception is FLS, for which six sequences are present, although it appears that only one encodes a functional enzyme (Owens et al., 2008). Because the products of the pathway are not essential for growth and development under greenhouse conditions, mutations in flavonoid genes are not lethal for the plant. Arabidopsis plants deficient in flavonoid metabolism are collectively referred to as transparent testa or (tt) mutants because of altered pigmentation in the seed coat, which is an easily identified phenotype (Shirley et al., 1995). These properties have made the pathway an excellent model for genetic and biochemical characterization.

The products of this pathway, which are derived from phenylalanine and acetyl CoA (Stafford, 1990), comprise a diverse set of compounds that perform a variety of physiological functions in the plant cell. Flavonoids are well known for their role as the major red, blue, and purple pigments in plants that are used to attract pollinators and seed dispersers (Weiss, 1991). These products also provide critical protection against UV radiation (Li et al., 1993), microbial infection, and herbivory (Stafford, 1990). Increasing attention has also been focused on the
antioxidant and anticancer properties of flavonoids with regard to human health (Harborne and Williams, 2000).

While the central steps of flavonoid biosynthesis are well detailed, full elucidation of the pathway is complicated by the fact that many of the enzymes are able to utilize several different substrates and may also engage in protein-protein interactions with each other (Burbulis and Winkel-Shirley, 1999; Winkel-Shirley, 2001). This raises the possibility that the pathway likely does not exist as a linear assembly of enzymes, but as a complex web in which interactions may occur as a result of, or in response to, a variety of factors. Because of opportunities for multiple interactions, there must exist mechanisms that control localization of these enzymes.

Until recently, it was thought that flavonoid biosynthesis occurred exclusively in the cytoplasm and that the products were then transported to sites of action in vacuoles, the cell wall, and the nucleus (Hrazdina and Wagner, 1985). However, there is now evidence that indicates that at least two of the enzymes, chalcone synthase (CHS) and chalcone isomerase (CHI), are also present in the nucleus suggesting that synthesis may occur in situ in some cases (Saslowsky et al., 2005). Recent work in our lab also suggests the presence of other flavonoid enzymes, AtFLS1 and AtDFR, are present in the nucleus of Arabidopsis mesophyll protoplasts when expressed from a constitutive promoter (unpublished data). Further studies are needed to explore the possibility that subcellular localization of flavonoid enzymes is affected by, or dependent upon, specific protein-protein interactions and that this localization determines the types and locations of end products that are produced in response to diverse biotic and environmental cues.

In order to explore the idea that different flavonoids have specialized functions depending on where they are located within the cell, studies were focused on determining the subcellular localization of CHS, and therefore the locations where flavonoids would be expected to accumulate. Confocal laser scanning microscopy was used to dissect the localization of CHS. Three-dimensional structures for several of these enzymes was used to explore which structural
components, including specific amino acids, affect localization of the enzymes and the accumulation of specific endproducts. Site-directed mutagenesis was used to alter key residues to determine their effects on localization of CHS and output of the pathway. This work represents an essential step toward elucidating the mechanisms that organize related metabolic enzymes within the cell and has applications to many other systems.
RESULTS

Expression of AtCHS::mGFP5 fusion under control of the CHS promoter

Previous work in our laboratory showed that CHS and CHI localize to both the cytoplasm and the nucleus of cells in the root tip and elongation zone (Saslowsky et al., 2005). These findings were based on the use of immuno-electron microscopy, immunofluorescence microscopy, subcellular fractionation, and localization of the GFP fusion proteins. GFP fusions offer a convenient reporter for studying the determinants of subcellular localization. However, the previous experiments using the GFP reporter gene relied on expression from the double-enhanced CaMV 35S promoter, which drives high-level ectopic expression that does not reflect the in-vivo situation for these enzymes. In order to retain temporal and spatial regulation of expression of AtCHS::mGFP5 for further localization experiments, a new construct was generated in which 1,328 bp upstream of the CHS translation start site (AtCHSp) were used to drive expression of the CHS coding region (AtCHScr) fused to mGFP5. This AtCHSp::AtCHScr::mGFP5 construct was introduced into plants homozygous for a new AtCHS null mutant allele in ecotype Columbia, tt4(020583), and ten independent lines were recovered.

In the absence of the transgene, seed produced from tt4(020583) plants lacked pigment in the seed coat (Figure 1B), consistent with the transparent testa phenotype of previously-identified alleles of this locus (Koornneef, 1990; Shirley et al., 1995; Saslowsky et al., 2000). The fusion protein was able to fully complement the seed coat phenotype in all ten lines containing the AtCHSp::AtCHScr::mGFP5 construct and to restore the production of anthocyanidins, visualized as purple pigmentation in the seedling hypocotyl (Figure 1C).
Visualization of stably-transformed plants using fluorescence microscopy indicated that the *AtCHS* promoter is extremely active in 4-day old seedlings. The promoter is sufficiently strong to drive levels that allow easy observation of GFP fluorescence in roots, with the most intense fluorescence visualized in the elongation zone. Localization patterns obtained by confocal laser scanning microscopy (CLSM) confirmed the presence of the *AtCHS*:mGFP5 fusion protein in the cytoplasm and nuclei of cells in the root elongation zone of these seedlings (Figure 2A). Nuclear localization was confirmed by DAPI staining (data not shown). The fusion protein was not detected in the meristematic zone of the root tip, unlike what was previously observed using constructs driven by the 35S promoter (Saslowsky et al., 2005). The relative position of the GFP fusion, C-terminal or N-terminal to *AtCHS*, did not affect on the localization patterns of CHS *in planta* (data not shown).

**Figure 1:** Complementation of *tt4* phenotype by *AtCHS* expressed as an mGFP5 fusion. *AtCHS* was expressed as an mGFP5 fusion from the native promoter in the *tt4*(020583) line. As seed coat color is a maternal phenotype, complementation is first observed in the T2 seed. Complementation of seedling pigmentation was examined in 3-day-old T2 plants grown on MS-sucrose. A) Wild type, B) *tt4*, C) CHS:mGFP5 in *tt4*, D) CHS<sup>R71A</sup>:mGFP5 in *tt4*, E) CHS<sup>NLSsub</sup>:mGFP5 in *tt4*. 
Effect of the R71A substitution on complementation and localization of CHS

*In silico* docking and molecular dynamics analysis of the AtCHS-AtCHI complex previously identified several residues predicted to be important for the interaction between AtCHS and AtCHI (Dana et al., *in revision*). AtCHS R71 participates in one of three salt bridges predicted to be important for this interaction. *In vitro* experiments using surface plasmon resonance (SPR) revealed that the interaction between AtCHS and AtCHI could be abolished by substituting R71 with alanine, although much of the enzymatic activity of the protein is retained (Dana et al., *in revision*). Interestingly, R71 is also part of a putative nuclear localization signal (NLS), R71K72R73H74 (Figure 3A)(Saslowsky et al., 2005).

**Figure 2:** Localization of AtCHS expressed as mGFP5 fusions from the native promoter in cells of the root elongation zone. Images are single optical sections taken from Z-stacks A) AtCHS::mGFP5, B) AtCHS$^{R71A}$::mGFP5, C) AtCHS$^{NLS_{sub}}$::mGFP5. Laser intensities were identical for A and B, but increased 2.5 fold for C.
To determine whether R$^{71}$ also plays a role in localization of CHS, site-directed mutagenesis was used to replace this residue with an alanine in the AtCHSp::AtCHS::mGFP5 construct. Ten independent $tt4$ lines were recovered that carried the modified construct. The AtCHS$^{R71A}$::mGFP5 fusion protein provided an intermediate level of complementation for seed coat color and proanthocyanidin production (Figure 1D), as well as reduced levels of GFP fluorescence compared to the unsubstituted fusion protein (Figure 2). Altered localization patterns were observed in single optical sections taken from Z-stacks, which showed clear nuclear localization for AtCHS::mGFP5, while AtCHS$^{R71A}$::mGFP5 appeared to surround the nucleus (Figure 2B). Additionally, AtCHS$^{R71A}$::mGFP5 accumulated at reduced levels in the cytoplasm of these cells.
Effects of the NLS substitution on complementation and localization

In order to evaluate the importance of the other three residues comprising the AtCHS NLS and their potential role in localization of CHS, site-directed mutagenesis was used to generate a construct in which the entire sequence, R\textsuperscript{71}K\textsuperscript{72}R\textsuperscript{73}H\textsuperscript{74}, was replaced with alanines. Eight independent tt4 lines were recovered that carried this $\text{AtCHS}^{\text{NLSub}}::\text{mGFP5}$ construct. This construct was unable to complement the tt4 phenotype (Figure 1E). One possible explanation for this outcome is that CHS must be localized to the nucleus for activation of its own promoter. To address this possibility, plants expressing a CHS promoter fusion, CHS1975:GUS (Feinbaum et al., 1991), was crossed into the tt4 (020583) background. F2 seedlings that showed no pigment production in the hypocotyl were identified at day 4 of development, indicating that they were homozygous for the tt4 allele and therefore contained no functional CHS protein. Histochemical staining for GUS activity showed that the CHS promoter was active in the tt4 background (Figure S2), an observation that was confirmed by semi-quantitative RT-PCR analysis of the transgene (Figure S3). This indicates that the presence of CHS is not required for the activation of the CHS promoter.

Further evidence that nuclear localization of CHS is not required for activation of its own promoter came from analysis of the transgenic lines for GFP expression, which showed that four of the eight $\text{AtCHS}^{\text{NLSub}}::\text{mGFP5}$ lines exhibited detectable fluorescence under UV light relative to controls. Interestingly, localization of this substituted fusion protein revealed substantially reduced levels of GFP fluorescence and abolished nuclear localization compared to $\text{AtCHS}::\text{mGFP5}$ and $\text{AtCHS}^{R71A}::\text{mGFP5}$. One possibility for this altered localization is that the substitutions destabilized the fusion protein and triggered a post-translational modification in which $\text{AtCHS}^{\text{NLSub}}$ was cleaved from GFP. However, immunoblot analysis using an anti-GFP antibody showed that the size of the expressed protein corresponded to that of the full-length fusion protein (Figure S4).
Another possible explanation for the lack of complementation by and nuclear localization of the introduced fusion protein is that AtCHS\textsuperscript{NLsub}::mGFP5 is not catalytically functional. Attempts to express the modified CHS as a thioredoxin fusion protein or as a GFP fusion in E. coli in order to determine its catalytic active were unsuccessful (Figure S5). Fukuma et al. (2007) recently showed that AtCHSR\textsuperscript{73} (Medicago R68) is completely conserved among the CHS superfamily of proteins, with only one exception. This residue is proposed to be critical for correct folding of the protein through its interaction with Q38 (Medicago Q33), as well as for correct positioning of the active site through its interaction with F220 (Medicago F215; Figure 2B)(Ferrer et al., 1999; Fukuma et al., 2007). Taken together, these results suggest that the inability of the CHS\textsuperscript{NLsub} fusion protein to complement the tt4 phenotype is due to effects on protein folding and stability. The work presented here represents the first \textit{in planta} evidence confirming \textit{in vitro} biochemical data and \textit{in silico} predictions for the functional roles of these specific residues.

\textit{Accumulation and distribution of flavonoid endproducts}

Analysis of flavonol content of the various transgenic plant lines was carried out in order to determine the effects of altered CHS localization on the accumulation and intracellular distribution of flavonoid endproducts. Flavonol extracts were prepared from whole 5-day-old seedlings as described in the materials and methods. HPLC chromatograms of extracts prepared from seedlings expressing the intact AtCHS fusion protein contained peaks with retention times corresponding to those of the flavonols, quercetin and kaempferol, at similar levels and ratios as in the wild type (Figure 4). Plants expressing AtCHS\textsuperscript{R71A} had reduced levels of kaempferol relative to quercetin (Figure 4D). The production of quercetin requires the activity of flavonoid 3’ hydroxylase (F3’H), an ER membrane-bound cytochrome P450 enzyme that should not be present in the nucleus. The elevated quercetin to kaempferol ratio is therefore
consistent with restriction of CHS\(^{R71A}\) to the cytoplasm, which could increase the overall production of quercetin. A small amount of what appears to be quercetin and no kaempferol were detected in plats expressing AtCHS\(^{NL\text{sub}}\) (Figure 4E).

![Figure 4: Flavonol content in transgenic lines. HPLC chromatograms extracted at 365nm are shown with peaks labeled Q (quercetin) and K (kaempferol). A) Wild type, B) tt4, C) CHS:mGFP5, D) CHS\(^{R71A}\):mGFP5, E) CHS\(^{NL\text{sub}}\):mGFP5](image)

To further examine the intracellular distribution of flavonoid endproducts, seedlings were stained with diphenylboric acid-2-aminoethyl ester (DPBA), a flavonol-specific histochemical fluorescence stain. DPBA-bound quercetin fluoresces orange-gold, while DPBA-bound kaempferol fluoresces green (Sheahan and Rechnitz, 1992; Buer and Muday, 2004). Accumulation patterns were obtained by CLSM. It was not possible to distinguish between kaempferol and quercetin using CLSM META linear unmixing due to difficulty obtaining specific emission spectra for each of the DPBA-bound flavonol compounds. Wild type DPBA-stained seedlings show clear cytoplasmic and nuclear accumulation of flavonols (Figure 5A). DPBA-stained flavonol synthase insertional plants containing a T-DNA insertion in 5′UTR of AtFLS1
(FLS1)(INRA AJ588535) accumulate nuclear and cytoplasmic flavonols (Figure 5B). These plants are known to accumulate reduced levels of quercetin and kaempferol (Owens et al., 2008). Strikingly, plants lacking F3’H (SALK_033945), which produce no quercetin, do not accumulate nuclear flavonols (Figure 5C). This suggests that the nuclear flavonols seen in wild type and the FLS1 null plants consists only of quercetin. DPBA staining patterns of tt4 plants expressing AtCHS::mGFP5 and AtCHS<sup>R71A</sup> ::mGFP5 were identical to wild type (data not shown). This differs somewhat from the results that Peer et al. (2001) have shown for flavonoid accumulation patterns in plasmolyzed roots. They report the nuclear accumulation of kaempferol in plasmolyzed roots of plants lacking F3’H. However, this dissimilarity could be due to differences between plasmolyzed roots versus intact roots or differences between the two F3’H alleles. Notwithstanding, this data suggests a potential role for quercetin, but not kaempferol, in the nucleus.

Figure 5: Flavonoid staining in roots using DPBA. A) Wild type, B) FLS1 null (INRA AJ588535), C) F3’H null (SALK_033945) accumulates only kaempferol. The absence of nuclear flavonols in plants lacking F3’H suggests that the nuclear staining observed in wild type and the FLS1 null plants is from quercetin, not kaempferol.
DISCUSSION

The co-localization of related enzymes to distinct subcellular sites is an established feature of metabolism that can, in at least some cases, contribute to the regulation of biochemical pathways (Wojtas et al., 1997; Graham et al., 2007). In addition to regulation at the transcriptional and translational level, modulating localization of enzymes provides an effective way for the cell to adjust metabolism depending on the products needed. Flavonoid metabolism provides us with an excellent model for dissecting the mechanisms that control the localization of related enzymes within the cell. The finding that CHS and CHI are localized, not only to the endoplasmic reticulum, but also to the nucleus in root cells was a surprising observation (Saslowsky et al., 2005). It suggests that not only the endproducts, but the enzymes themselves have roles in the nucleus, although these remain to be identified. The major goal of this study was to clearly define the localization patterns of CHS within root cells, to begin to dissect its roles in different subcellular compartments, and to determine the contributions of specific amino acid residues to this differential localization.

The previous observation of CHS in the nuclei of root cells was recapitulated in this study and localization patterns were examined in further detail. The native promoter was sufficiently active to drive expression of the CHS::mGFP fusion to detectable levels. Further, the CHS fusion protein was found to be absent from the meristematic zone of the root tip in four-day-old seedlings, consistent with analysis of promoter activity using the GUS reporter. Clear nuclear and cytoplasmic localization patterns were obtained in the differentiation zone of roots. As mentioned above, there is now evidence for the presence of other flavonoid enzymes in the nucleus from experiments with additional fluorescent protein fusions (Kevin Crosby and Brenda Winkel, unpublished data). The intriguing possibility remains that different subsets of the flavonoid machinery are found at distinct locations in the cell and are dedicated to producing specific endproducts.
The use of site-directed mutagenesis to determine the specific roles of key amino acids had several unanticipated outcomes. The R\textsuperscript{71} substitution altered the localization of CHS and decreased the overall complementation of the tt4 phenotype. This residue is known to be critical for the interaction between CHS and CHI. Structural analysis of CHS predicted that R\textsuperscript{73} is a critical residue for CHS folding and stability. This prediction is supported by the fact that substitution of the putative NLS completely abolished localization and the ability of the fusion protein to complement the tt4 phenotypes. The work presented here represents the first in planta evidence confirming in vitro biochemical data and in silico predictions for the functional roles of these specific residues. These findings also suggest that CHI could impact the stability and activity of CHS through the interaction at R\textsuperscript{71}. There is a precedent for proteins modulating the stability of partners (Serdiuk, 2007) and this possibility represents largely unexplored aspect of flavonoid metabolism.

The analysis of flavonol profiles from the modified CHS fusion proteins suggests potential roles for F3’H in the accumulation of quercetin and kaempferol. The altered ratio of quercetin to kaempferol is consistent with restriction of CHS\textsuperscript{R71A} to the cytoplasm, which could increase the overall production of quercetin. DPBA-stained atfls1 insertional knockout plants accumulate nuclear and cytoplasmic flavonols. These plants are known to accumulate reduced levels of quercetin and kaempferol, possibly due to compensation by anthocyanidin reductase (ANS)(Owens et al., 2008). The fact that atf3’h insertional plants accumulate no nuclear flavonoids under the conditions tested suggest a role for quercetin, but not kaempferol, in the nucleus.

However, the specific role of flavonoids and flavonoid enzymes in the nucleus remains unclear. Flavonoids are known to interact with proteins and nucleic acids (Sarma and Sharma, 1999; Ramadass et al., 2003). The reactive nature of the compounds suggests that their presence in the nucleus must be tightly monitored. There is evidence that in some cases flavonoids can modulate gene expression (Hodek et al., 2002). Flavonoids are also known
negative regulators of the plant hormone auxin (Jacobs and Rubery, 1988; Brown et al., 2001). In roots, flavonoids are known to modulate auxin transport and have a role in the gravitropic response (Buer and Muday, 2004). It is thus possible that flavonoids or the flavonoid enzymes themselves play a role in modulating gene expression in response to developmental or environmental cues. A growing number of metabolic enzymes are known to have “moonlighting” functions, including as transcription factors in the nucleus (Moore, 2004) and the function of CHS in the nucleus may well be unrelated to its previously-defined catalytic role. However, the possibility that CHS regulates its own gene expression or the expression of CHI was ruled out in this study. The function of these enzymes in the nucleus remains an area of substantial interest for future work.
MATERIALS AND METHODS

Plant material and growth conditions

*Arabidopsis* wild type, mutant, and transgenic lines (all Columbia ecotype) were grown in Promix BX soil supplemented with Osmocote controlled release fertilizer (Scotts Inc., Marysville, OH) at the manufacturer’s recommended concentration. For imaging and analysis of gene expression and flavonol accumulation, seedlings were grown on the surface of agar medium containing Murashige and Skoog salts and sucrose under continuous white light (100 µE m⁻²s⁻¹) at 21°C as described previously (Saslowsky and Winkel-Shirley, 2001).

Segregating T-DNA insertional mutant lines (Alonso et al., 2003) for *AtCHS* (At5g13930), SALK_020583, and for *AtF3’H* (At5G07990), SALK_033945, were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). Homozygotes were initially identified based on altered pigmentation in the seed coat, as described previously for mutations in these genes (Koornneef, 1990). Both lines contain a T-DNA insertion within the second exon of the corresponding gene (Figure S1). The presence of the transgene was confirmed by extracting genomic DNA from wild type and putative mutant lines using a miniprep procedure (Edwards, 1991) followed by PCR amplification using gene-specific primers (Integrated DNA Technologies, Table S1) with or without the left-border primer, Lba1 (Alonso et al., 2003) (Figure S1).

Construction of transgenic arabidopsis plants expressing green fluorescent protein fusions

MultiSite Gateway® Technology was used to construct multi-fragment clones containing a 5’ promoter region for CHS, the CHS coding region, and a 3’ region containing the mGFP5
reporter gene. All amplification reactions were performed using PfuUltra polymerase (Stratagene) with primers (Integrated DNA Technologies) designed to introduce the appropriate TOPO or att sites (Table S2). The AtCHS promoter region, defined as the 1,328 bp between the stop codon of the upstream gene and the start codon of AtCHS, was amplified from genomic wild type (Col) DNA and inserted into the pDONR P4-P1R vector using the BP recombination reaction (Invitrogen). The CHS coding region was amplified from a modified pET32a(+) vector (Novagen) containing the coding region from the Landsberg erecta ecotype (Dana et al., in revision). The fragment was inserted into the pENTR/SD-TOPO vector using the pENTR™ Directional TOPO® Cloning Kit (Invitrogen). The coding sequences for mGFP5 was amplified from pAVA393 (von Arnim et al., 1998) and inserted into the pDORN P2R-P3 vector using the BP recombination reaction (Invitrogen). The integrity of the resulting entry clones was confirmed by sequencing.

For the generation of transgenic plants, the three entry vectors were inserted into the plant binary destination vector, pB7m34GW (Flanders Institute for Biotechnology, Ghent University; (Karimi et al., 2005), using the MultiSite Gateway® LR reaction (Invitrogen). The resulting multi-fragment constructs were used to transform Agrobacterium tumefaciens strain GV3101 using a freeze-thaw method (Chen et al., 1994) and then introduced into Arabidopsis plants using the floral dip method (Clough and Bent, 1998). Selection of transformants was carried out on T1 seedlings grown on soil on days 7 though 12 and days 15 through 20 after sowing by spraying once each day with a 1:10,000 dilution of glufosinate ammonium concentrate (Basta; AgrEvo Co.), 0.005% Silwet L-77 (Lehle) per the manufacturer’s instructions (Frazzon et al., 2007). Genomic DNA was extracted from putative transformants (Edwards, 1991) and analyzed by PCR using primers specific for the transgene.
**Site-Directed mutagenesis of AtCHS**

Site-directed mutagenesis was carried out using the QuikChange system (Stratagene) and the primers listed in Table 3. The pENTR entry clone containing the AtCHS coding region was modified to substitute R\textsuperscript{71} with alanine to generate pENTR-CHS\textsuperscript{R71A}. This plasmid was used as the template to further substitute K\textsuperscript{72}R\textsuperscript{73}H\textsuperscript{74} with alanines to generate the full putative nuclear localization signal (NLS) substitution construct, pENTR-CHS\textsuperscript{NLSsub}. The integrity of the modified plasmids was confirmed by restriction digestion and sequencing.

**Transgene RNA expression analysis**

RNA was extracted from confirmed transgenic plant lines using Tri Reagent (Sigma-Aldrich) per the manufacturer’s recommendations. The RNA was treated with TURBO-DNase (Ambion) and the concentration was estimated based on the absorbance at 260 nm measured using a NanoDrop Spectrophotometer ND-1000. cDNA was prepared from up to 2 µg of treated RNA using an oligo d(T) primer (Invitrogen) with an Omniscript Kit (Qiagen). Semi-quantitative RT-PCR was performed using the cDNA as a template with AtCHS specific primers (Table S2)(Integrated DNA Technologies). Primers specific for the AtActin2 gene were used as a positive control (Frazzon et al., 2007).

**Flavonoid / histochemical staining and imaging of live seedlings**

Flavonoid staining with DPBA was carried out on 4-day-old seedlings as described by Buer and Muday (2004). GUS activity was assayed in 4-day-old seedlings using a modified procedure previously described in Owens et al. (2008). For localization studies, 5-day-old seedlings were analyzed by using a LSM510 (Carl Zeiss) confocal laser scanning microscope.
with a 1.2 numerical aperture 40X C-Apo water-immersion objective lens, 488 nm argon laser line, and a 505-550 band pass filter as described in Saslowsky et al. (2005) or a 505 long pass filter.

**Recombinant protein expression and purification**

Recombinant CHS was expressed from the modified pET32a(+) vector (Novagen), pCD1, as described previously (Dana et al., *in revision*). A pCD1-CHS<sub>NLSsub</sub> expression construct was generated by amplifying the CHS coding region from pENTR-CHS<sub>NLSsub</sub> using PfuUltra (Stratagene) and subcloning this fragment into the Ncol and HindIII sites of pCD1-CHS, digesting the latter with EcoRI to reduce the chances of re-ligation of the liberated fragment. The digested PCR product and vector were combined in a 3:1 molar ratio with T4 ligase (New England Biolabs). The ligation reaction was used to transform electrocompetent *E. coli* DH10B cells. The presence of the insert in the resulting clone was confirmed by PCR and restriction digestion, and the integrity of the insert was confirmed by sequencing. The construct was then used to transform *E. coli* BL21(DE3)pLysS cells (Invitrogen) using a heat shock protocol, according to the manufacturer's instructions.

For recombinant protein expression, cells were grown overnight at 37°C in LB containing 100 µg/ml ampicillin and 25 µg/ml chloramphenicol, then diluted into one liter of the same medium and allowed to grow until mid-log phase (OD<sub>600</sub> of 0.4). Expression was induced by the addition of 1mM isopropyl-β-D-thiogalactopyranoside (Anatrace) and growth for a further 4 h at room temperature. Cells were harvested by centrifugation (5000 rpm for 15 min at 4°C) and the pellets stored at -80°C. The cells were lysed in buffer containing 10 mM HEPES, 150 mM NaCl, 10% glycerol, and 0.1% Tween 20. The sample was then sonicated on ice for 45 sec and allowed to recover for 1 min. This was repeated four times. The sample was centrifuged at
15,000 rpm for 50 min at 4°C to pellet cellular debris. The supernatant was then decanted and sterilized by filtration through a 0.45µm cellulose acetate membrane (Corning). The sample was injected into an AKTA FPLC (Amersham Biosciences) and the fraction containing the purified recombinant protein was identified in the eluate by a peak in absorbance at 280 nm. The fraction was collected and dialyzed overnight at 4°C in HEPES buffered saline (10 mM HEPES, 150 mM NaCl). The concentration of the recombinant protein was estimated by comparison to BSA standards of known concentrations on a SDS-PAGE gel.

**Analysis of flavonol profiles**

Five-day-old seedlings were harvested and flash-frozen in liquid nitrogen. Flavonols were extracted according to Owens et al. (2008). Analysis was performed using a Waters HPLC system with a 2996 photodiode array and Empower 2 software as described previously by Owens et al. (2008). Chromatograms were extracted at 365 nm.

**Protein model visualization**

DeepView/Swiss-Pdb Viewer v.3.9 was used to map residues of interest on a homology model for AtCHS that was previously generated based on the crystal structure of *Medicago sativa* CHS2 [pdb id: 1B15](Dana et al., 2006).
### SUPPLEMENTAL MATERIALS

#### Table S1: Primers used for identification of homozygous T-DNA insertion lines

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<th>Target</th>
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<td>SALK_020583</td>
<td>CHS T-DNA forward</td>
<td>GATCACTCATGTCGTCTTCTG</td>
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<td>SALK_020583</td>
<td>CHS T-DNA reverse</td>
<td>TTAGAGAGGAACGCTGTGC</td>
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<tr>
<td>SALK_033945</td>
<td>F3’H T-DNA forward</td>
<td>CAGCGGATTTGAATTTGAAC</td>
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<tr>
<td>SALK left boarder</td>
<td>Lba1</td>
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#### Table S2: Primers used for generation of Multi-Fragment Gateway constructs

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<td>CHS promoter</td>
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<td>promCHI-forward</td>
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<td>CHI promoter</td>
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<td>CHS coding</td>
<td>CHS coding pENTR forward</td>
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</tr>
<tr>
<td>CHS coding (no stop)</td>
<td>CHS coding pENTR reverse</td>
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<tr>
<td>CHS coding (stop)</td>
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<td>CGTCAGATCGCCGCCGCCGCAATTG</td>
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*The altered codon is underlined.

**Table S3:** Primers used for CHS site-directed mutagenesis

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<th>Primer Sequence*</th>
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<tr>
<td>CHS 3’ end</td>
<td>CHS 3’ reverse</td>
<td>TTAGAGGAACGCTGTGC</td>
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<td>CHI 3’ end</td>
<td>CHI 3’ forward</td>
<td>ACCGGGATCGCTGTAGTCCAG</td>
</tr>
<tr>
<td>CHI 3’ end</td>
<td>CHI 3’ reverse</td>
<td>GTTGTCTTTGGCTAGTTTTT</td>
</tr>
<tr>
<td>mGFP5 3’ end</td>
<td>mGFP5 3’ forward</td>
<td>TACCAGACAACATTACGTGTTAAC</td>
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<tr>
<td>mGFP5 3’ end</td>
<td>mGFP5 3’ reverse</td>
<td>AGCTTTGATAAGTCATTCATGCCCAT</td>
</tr>
<tr>
<td>AtActin2</td>
<td>AtActin2 forward</td>
<td>TCGGTTGTCCATTTGCT</td>
</tr>
<tr>
<td>AtActin2</td>
<td>AtActin2 reverse</td>
<td>CTCTCAACATCAAAGCGCTTAAAAG</td>
</tr>
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</table>

*Uppercase letters represent the recombination site. Lowercase letters are gene-specific.
Figure S1: T-DNA insertional knockout lines used in this study. Two new \( tt \) alleles were initially identified based on altered seed coat color. A) SALK_020583 contains a T-DNA insertion within the second exon of CHS. B) SALK_033945 contains a T-DNA insertion within the second exon of F3’H. Arrowheads indicate the orientation of the left border of the T-DNA. Homozygotes were confirmed by PCR analysis of genomic DNA using the primers listed in table S1.
Figure S2: CHS promoter activity in tt4 background. Plants expressing a CHS promoter fusion, CHS1975:GUS (Feinbaum et al., 1991), were crossed into the tt4(020583) background. Histochemical staining for GUS activity (Owens et al., 2008) showed that the CHS promoter is active in plants lacking CHS protein.
Figure S3: Semi-quantitative RT-PCR of transgenic plant lines. RNA was extracted from whole 5-day old seedlings. Gene targets are listed on the left in italics. Positive controls (+) contained plasmid DNA, negative controls (-) contained no template.
Figure S4: Immunoblot of whole seedling extracts probed with anti-GFP antibody (gift from William W. Ward at Rutgers University). Total protein was extracted from one or ten seedlings and separated by SDS-PAGE. A band of approximately 73 kDa (indicated by the arrow), corresponding to the anticipated size of the AtCHS::mGFP5 fusion protein, can be visualized in samples prepared from transgenic seedlings.
Figure S5: Expression of recombinant CHS in E. coli. Recombinant CHS (native and modified) was isolated from E. coli and purified by FPLC. TRX::CHS was efficiently expressed in E. coli, while TRX::CHS NLSub was not. Further, the fusion of mGFP5 to the C-terminus of CHS NLSub did not enhance the in vitro expression of the recombinant protein.
ACKNOWLEDGMENTS

The authors would like to thank Kristi DeCourcy for lending her expertise in confocal microscopy. They also acknowledge the Arabidopsis Biological Resource Center for providing the T-DNA insertion lines used in this study. This work was supported by the National Science Foundation (grant MCB-0445878), the Graduate Research and Development Project of Virginia Tech, and the Department of Biological Sciences at Virginia Tech.
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Chapter 4

Summary
The work described in this dissertation was focused on two metabolic pathways, one that is ubiquitous among living organisms and another that is entirely unique to plants. The major aim of this work was to examine the organization of each system in an attempt to evaluate underlying mechanisms of regulation that are fundamental features of all metabolic pathways.

The assembly of [Fe-S] clusters involves an ancient biosynthetic system that provides critical function to proteins that have roles in numerous life-sustaining processes. This system is well detailed in bacteria, yeast, and mammals, yet surprisingly little is known about how this machinery works in plants. The intriguing possibility remains that subsets of [Fe-S] cluster machinery function at distinct intracellular locations or organelles. There is certainly a precedent for this in humans where the IscU1 and IscU2, homologous to yeast Isu1 and Isu2, localize to the cytosol and mitochondria, respectively (Tong and Rouault, 2000). Plants provide a unique system for exploring this idea because they contain chloroplasts, a potential location for yet another distinct [Fe-S] cluster assembly machinery. Therefore, the goal of this study was to apply what had previously been defined in bacteria, yeast, and humans, to the virtually unexplored system present in plants. The work was focused on making the first characterizations of what aspects of this ancient system were conserved among all organisms, as well as how the system was modified to fulfill the unique requirements of plant cells.

This study offers the first functional characterization of genes involved in mitochondrial [Fe-S] cluster assembly in Arabidopsis. Through the collaborative efforts of the authors, the first biochemical evidence for the cysteine desulfurase activity of AtNFS1 was obtained. Additionally, AtNFS1 was shown to participate in cluster assembly with a predicted Arabidopsis scaffold protein, AtISU1 in vitro. The predicted mitochondrial localization for three proteins involved in [Fe-S] assembly was confirmed.
The majority of the work contributed by myself was focused on evaluating the phenotypic
effects of altered expression of $AtNFS1$ and $At\ ISU1$-3. The importance of $AtNFS1$ and
$At\ ISU1$-3 can be demonstrated by the severe phenotypic effects of downregulation.
Further, it was not possible to obtain plants with a homozygous insertion for $At\ NFS1$.
This provides compelling evidence that $AtNFS1$ functions as the sole cysteine
desulfurase for $[Fe-S]$ cluster assembly in mitochondria. Taken together, these results
provide a solid foundation for further studies, as many of the details of $[Fe-S]$ cluster
assembly in plants remain unknown.

In contrast to the machinery for $[Fe-S]$ cluster assembly, flavonoid biosynthesis is
one of the best-characterized plant metabolic pathways. The substantial body
information regarding flavonoid biosynthesis has established this pathway in $Arabidopsis$
as a model system for studying enzyme complexes. Therefore we are able to now direct
our attention towards exploring the organization of these related metabolic enzymes in
the flavonoid pathway.

The use of enzyme complexes as a way to enhance the efficiency of metabolism
is well established (reviewed in Srere, 2000; Winkel, 2004). This organization allows for
the accumulation of high local substrate concentration, the direct channeling of
compounds between active site, and the separation of toxic intermediates away from the
bulk of the cell. What remain to be established are the underlying molecular
mechanisms that control the assembly of these complexes. Mounting evidence for the
highly crowded environment of the cell interior emphasizes the need for highly ordered
and fundamental mechanisms to regulate and maintain cellular metabolism.

The localization of enzymes has been shown to be one way that the cell can
regulate metabolism (Srere and Knull, 1998). By modulating localization, the cell can
control the access of a protein to potential interacting partners. While the observation of
CHS and CHI in the nucleus is surprising (Saslowsky et al., 2005), it suggests that
these proteins have physiological roles in this organelle. The goal of this study was to define the determinants of the nuclear localization of CHS and to explore the physiological effects of altered localization in order to begin to define the role of CHS in the nucleus.

This study is one of the first focused on determining the localization of flavonoid enzymes in Arabidopsis. Further, this is the only study to date that observes the localization of CHS, expressed at physiologically-relevant levels, in live Arabidopsis seedlings. Therefore, this work presents the most accurate data yet for defining the localization of CHS.

The presence of CHS in the nucleus suggests the possibility of dual machineries located in distinct organelles and dedicated to providing specific end products. The flavonoid accumulation patterns observed in this study point to the fact that the presence of specific enzymes affect not only the complement of endproducts being produced, but the location at which these endproducts accumulate. The possibility also remains that there is a novel physiological role for CHS in the nucleus. An increasing number of metabolic enzymes are now shown to have “moonlighting” functions, including roles as transcription factors (Moore, 2004) and it is possible that the presence of CHS in the nucleus is unrelated to its previously defined role. However, the possibility that CHS regulates the expression of its own gene or that of CHI was ruled out in this study.

Another goal of this work was to determine the role of specific amino acid residues in the localization of CHS. Modification of R$_{71}$ altered the nuclear localization of CHS in root cells and the fusion protein appeared to accumulate around the nucleus. Modification of the entire NLS has unforeseen effects on the stability of the CHS fusion protein. In silico and in vitro studies predict a role for R$_{73}$ through its interaction with Q38 (Medicago Q33) and F220 (Medicago F215) for correct folding and correct positioning of the active site, respectively (Fukuma et al., 2007). This region of CHS, though distal
from the active site, is clearly a critical part of the protein. This work represents the first
evidence confirming *in vitro* biochemical data and *in silico* predictions for the functional
roles of this residue in *Arabidopsis*.

Another way to evaluate the role of the nuclear localization of CHS is to
sequester CHS away from the nucleus using a nuclear export signal (NES). Transgenic
plants expressing CHS and a GFP<sup>NES</sup> fusion in the *tt4* background have already been
generated are the T1 seed is now ready to be analyzed. It should be noted that nuclear
export signals are not clearly defined in plants and it might not be possible to completely
restrict CHS from the nucleus. If feasible, it will be interesting to determine whether the
CHS-NES fusion protein is able to complement the *tt4* phenotype if it is excluded from
the nucleus.

The work focused on defining the roles of specific amino acids residues of CHS
also provided information regarding the use of current methodologies for modulating
protein function. Site-directed mutagenesis is the current gold standard for studying the
relationship between structure and function of proteins. However, there can be
unforeseen effects, distal from the modification that can make obtaining meaning results
difficult (Dunham et al., 2003; Fukuma et al., 2007). Therefore, site-directed
mutagenesis should not be viewed as a singular alteration to a particular residue, but as
an alteration to the protein as a whole.

Work from our lab currently supports the idea that CHS functions as a hub for
interactions with numerous other proteins with functions both within and outside of the
pathway (Watkinson, Crosby, Bowerman, Howard, and Winkel, unpublished
data)(Burbulis and Winkel-Shirley, 1999; Owens et al., 2008; Owens et al., 2008)).
Studies devoted to uncovering global interactomes for organisms suggest that while
most enzymes will have a limited number of possible partners, a small subset of these
proteins will have multiple partners (Moore, 2004). CHS is a likely candidate for this
latter function as it has been shown to interact with a number of other flavonoid enzymes (Burbulis and Winkel-Shirley, 1999), but only recently are the interfaces of these interactions being defined (Bowerman and Winkel, unpublished data). Other work from our lab supports the idea that there might be competition for binding among these enzymes. FLIM-FRET studies show that DFR and FLS1 compete for interaction with CHS (Crosby and Winkel, unpublished data). In addition to F3'H, which functions as a membrane anchor for the pathway in the cytoplasm, CHS could function as a hub for the formation of flavonoid metabolons elsewhere in the cell. Current work is focused on determining whether there are distinct flavonoid metabolons at various locations in the cell aimed at producing different flavonoid endproducts and identifying the specific proteins present in these complexes. Our lab is also in the process of identifying proteins that interact with CHS, but that have functions unrelated to flavonoid biosynthesis (Watkinson, Howard, and Winkel, unpublished data) to explore the possibility that CHS functions as a link between flavonoid metabolism and other metabolic pathways.

The work presented in this dissertation provides further evidence that the organization of metabolism is an essential feature of cellular biochemistry. The pathways of [Fe-S] cluster assembly and flavonoid biosynthesis serve as excellent models for genetic and biochemical studies devoted to studying metabolic organization and work on these systems is likely to continue making substantial contributions to our overall understanding of the mechanisms that coordinate related enzymes in plant metabolism.
LITERATURE CITED


Isolation and transfection of leaf mesophyll protoplasts from *Arabidopsis*

This following protocol was optimized for use in the Winkel lab and is based on the protocol developed by Sheen (2002).
Protoplast isolation and transfection

Isolation and transfection of leaf mesophyll protoplasts was performed as described in Sheen (2002), with minor modifications, as noted below. The procedure was intended to be used as a transient expression system to test the efficacy of the plasmid constructs used in Chapter 3. Protoplasts were prepared using leaves from 4 to 6-week-old Arabidopsis plants. The leaves were cut into 0.5-1 mm strips and incubated in an enzyme solution containing 1% cellulose, 0.2% macerozyme R10 (Yakult Honsha, Tokyo, Japan), 0.4 M mannitol, 20 mM KCl, 20 mM MES at pH 5.7. The solution was heated at 55°C for 10 min, filtered sterilized through a 0.45 µm nylon membrane, and supplemented with 0.1% BSA and 10 mM CaCl₂. The leaf strips were vacuum infiltrated until saturated and left to digest in the enzyme solution in the dark for 3 h. The solution containing the resulting protoplasts was filtered through 70 µm nylon mesh into a round bottom tube and pelleted at 100 x g for 1-2 min. The protoplasts were washed once in cold washing and incubation (WI) solution containing 0.5 M mannitol, 20 mM KCl, 4 mM MES, pH 5.7 and finally resuspended in W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 2 mM MES, pH 5.7). After a 30 min incubation on ice, the protoplasts were pelleted and resuspended in 1 ml MMg solution (0.4 M mannitol, 15 mM MgCl₂, 4 mM MES, pH 5.7) in preparation for transfection.

Transfection was carried out by adding 10 µg of CsCl₂ purified plasmid DNA (Ish-Horowicz and Burke, 1981) to 100 µL of the protoplast suspension, adding an equal volume of PEG/Ca solution (40% v/v PEG400 in 0.8 M mannitol/1 M CaCl₂), and then incubating at room temperature for 5 to 30 min. The sample was then diluted with twice the volume of W5 solution and mixed gently. The protoplasts were pelleted, resuspended in 50 µL W5 solution. A variety of incubation conditions were tested to
determine the optimal conditions for expression from the native CHS promoter. Protoplasts remained healthy and intact when incubated for 16 h. at room temperature. These incubation conditions also provided detectable levels of the GFP reporter in constitutively driven control samples.

Transfected protoplast were suspended on glass slides between to glass coverslips and another glass slide was placed on top of the sample, supported by the coverslips on either side. This method of slide preparation prevented the lysing of protoplasts. Samples were visualized using a 40x objective on a Zeiss Axiovert 200 fluorescent microscope.

Although this transient expression system using Arabidopsis protoplasts provides a quick option for testing transgenes, it was unsuitable for our needs. It was not possible to express our transgenes in this system using the native CHS promoter. This could be due to the fact that CHS expression is not highly expressed in Arabidopsis mesophyll cells at the 4 to 6-week old stage. It could also be due to the fact that the activity of the CHS promoter depends on biological cues that are not maintained when the plant is plasmolyzed. Although this system remains a valid tool for evaluating expression and localization of transgenes, it should be substantiated with analysis of stably-transformed plants when possible.
Figure 1: Transient mGFP5 expression in *Arabidopsis* mesophyll protoplasts. Protoplasts were harvested from healthy leaf material and transfected with a 35S::mGFP5 construct. A) Light image, B) FITC image. Images were taken with a 40x objective on a Zeiss Axiovert 200.
LITERATURE CITED


APPENDIX II

In vitro assay for chalcone synthase enzyme activity

This following protocol was optimized for use in the Winkel lab and is based on the protocol developed by Jez et al. (2000).
**CHS in vitro activity assay**

This assay was performed as described by Jez et al., (2000) with modifications, as noted below. The modified procedure was intended for use as a method for determining the *in vitro* activity of modified CHS proteins developed in Chapter 3. The CHS activity assay described by Jez et al. (2000) was used as a the foundation from which this assay was developed. The assay set up was identical except that it was not carried out using radiolabeled isotopes. The method for monitoring the progression of the assay was developed for our purposes.

The CHS substrate, coumaroyl-CoA, was synthesized from *p*-coumarate and coenzymeA lithium salt (Sigma) using recombinant tobacco 4-coumarate-CoA ligase (produced in *E. coli* from a clone provided by Eran Pichersky, University of Michigan) according to the method of Beuerle and Pichersky (2002). The concentration of coumaroyl-CoA was calculated using the following calculation:

\[
\text{Abs}_{333}/\text{extinction coefficient} \times \text{dilution factor} = [ ]
\]

Reactions were carried out using 10 µg of recombinant protein, 0.2 mg BSA, 0.1 M KPO₄, pH 7.0, 80 µM malonyl CoA, and 40 µM coumaryl CoA. The total volume was brought to 1 ml with HEPES buffered saline (150 mM NaCl, 10 mM HEPES, pH 7.0). A negative control contained recombinant protein that had been boiled for 10 min. The reactions were set up without the protein and used to blank the spectrophotometer. As soon the protein had been added, the first time point (T0) was taken. The reaction was monitored every 2 min for the first 10 min and then every 1 min thereafter. Progression of the reaction was measured as a loss of substrate as indicated by a decrease in absorbance at 333 nm. The resulting specific activity values, calculated as µmoles of substrate converted per µg of chalcone synthase per second, were plotted against time in minutes.
LITERATURE CITED
