DISSERTATION

THE ROLE OF CPNIFS IN SELENIUM AND SULFUR PLANT METABOLISM: IMPLICATIONS FOR PHYTOREMEDIATION AND PHOTOSYNTHESIS

Submitted by
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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY DOUG VAN HOEWYK ENTITLED "THE ROLE OF CPNIFS IN SELENIUM AND SULFUR PLANT METABOLISM: IMPLICATIONS FOR PHYTOREMEDIATION AND PHOTOSYNTHESIS" BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

THE ROLE OF CPNIFS IN SELENIUM AND SULFUR PLANT METABOLISM: IMPLICATIONS FOR PHYTOREMEDIATION AND PHOTOSYNTHESIS

NifS-like proteins are a conserved group of proteins that can cleave the sulfur-containing amino acid cysteine in alanine and elemental sulfur (S), and selenocysteine into alanine and selenium (Se). In yeast and bacteria, NifS-like proteins are essential for survival because they provide the S for iron(Fe)-S clusters, a prosthetic group that is inserted into various FeS proteins that have a role in electron transfer. Furthermore, NifS-proteins are an essential part of Se metabolism in organisms that require this trace element. The goal of this research was to characterize the function of a chloroplastic NifS-like protein in *Arabidopsis thaliana*, designated AtCpNifS. As described in this dissertation, overexpression of CpNifS increases plant tolerance to selenate and accumulation of Se. Increased levels of CpNifS prevents toxic incorporation of selenocysteine into proteins, and thus enhances Se tolerance. This may benefit phytoremediation- the use of plants to naturally clean polluted soils and groundwater. In an effort to further the field of phytoremediation, a transcriptome experiment was performed in order to identify other genes and pathways that are involved in responding to Se stress. However, as divulged, plants likely do not require Se for essential metabolism, and the true function of CpNifS is more likely in the maturation of FeS clusters. The knockdown of CpNifS proteins in Arabidopsis using an inducible RNAi approach revealed that chloroplast function and structure became impaired, and that levels of all tested FeS proteins decreased. Consequently, the rate of photosynthetic electron transport, which is dependent on FeS proteins , diminished, and plants became chlorotic and eventually died. Therefore, CpNifS is required for FeS proteins, and is essential for proper photosynthesis and plant growth.

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Chapter 1: Introduction and the scope of the dissertation

NifS-like proteins are a conserved group of proteins that can cleave the sulfur-containing amino acid cysteine into alanine and elemental sulfur (S), and selenocysteine into alanine and selenium (Se). Therefore, NifS-like proteins can be described as having both cysteine desulfurase (CysD) activity and selenocysteine lyase (SL) activity. In yeast and bacteria, the CysD activity of NifS-like proteins is essential for survival because it provides the S for iron (Fe)-S clusters, a prosthetic group that is inserted into various FeS proteins that have a role in electron transfer. Furthermore, NifS-proteins are an essential part of Se metabolism in organisms that require this trace element.

Plants contain three NifS-like proteins. The mitochondrial (mt)NifS-like protein is homologous to IscS in *E.coli*. A cytosolic protein designated ABA3 has a NifS-like domain that likely is specifically involved in the biosynthesis of molybdenum cofactor. The chloroplastic (cp)NifS is most similar to SufS in *E.coli*. In analogy to SufS, CpNifS contains high SL activity: its SL activity is nearly 300x higher than its CysD activity, which is intriguing in view of the current thought that plants do not require Se for survival. The discovery that plants contain a NifS-like protein localized to the chloroplast prompted the question of whether or not this protein has unique properties and functions in plant development.

In the past five years the study of CpNifS has been studied extensively *in vitro* by my colleague Hong Ye. I have studied the roles of CpNifS *in vivo*, using transgenic and genomic approaches. What exactly is the role of CpNifS, and can its function be complemented by MtNifS? What is the function of the high SL activity of CpNifS, and are there any ramifications if CpNifS is overexpressed in Arabidopsis plants? Is CpNifS essential for the maturation of FeS proteins in both the chloroplast and mitochondria? These questions, as well as several related ones, are addressed in this dissertation.

Chapter Two, entitled "The functions of NifS-like proteins in plant sulfur and selenium metabolism" provides the reader with a background on NifS-like proteins in plants, with a detailed emphasis on the already known, and possible additional functions of CpNifS. The effect of CpNifS overexpression in *Arabidopsis thaliana* is addressed in Chapter Three. Selenium manifests its toxicity when selenocysteine (SeCys) is

mistakenly incorporated into protein. Because CpNifS cleaves SeCys and prevents its incorporation into protein, I questioned if its overexpression would increase tolerance to selenate in plants. I further wanted to know if CpNifS overexpression would (a) enhance Se accumulation, (b) decrease the amount of Se in protein, and (c) affect the sulfur (S) levels of plants grown with or without Se.

Chapter Four describes the effects of selenate on the transcriptome of Arabidopsis roots and shoots. While it was important to identify specific genes that were differentially regulated by Se, a broader focus was kept in view by trying to understand which plant processes and pathways were responding to Se. This study of how genes in root and shoot tissue respond to Se stress was followed up by biochemical and genetic studies to obtain further insight into Se tolerance mechanisms in Arabidopsis.

Chapter Five explores the role of CpNifS in FeS protein maturation. In this last chapter, levels of CpNifS were knocked down using an inducible RNAi approach, to better understand the function of CpNifS in plants. This study was aimed to illustrate the changes that occur in the physiology and development of the plant as a result of decreased levels of CpNifS. Will decreased levels of CpNifS affect FeS containing proteins, and have a concomitant effect on FeS-dependent cellular processes, such as electron transport during respiration and photosynthesis?

The experimental chapters are followed by a summarizing discussion of the dissertation, which reflects upon some of the broader implications of the findings and addresses future experiments that may shed more light on our understanding of Se metabolism in plants. Finally, in the Appendix some preliminary results are presented that may be followed up by others.

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Review

The functions of NifS-like proteins in plant sulfur and selenium metabolism

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Abstract

NifS-like proteins were originally studied in bacteria, where they play an important role in sulfur (S) and selenium (Se) metabolism. NifS-like proteins, now thought to exist in all organisms, are best known for their cysteine desulfurase activity that catalyzes the conversion of cysteine into alanine and elemental S needed for various cofactors: iron–sulfur clusters, thiamine, biotin and molybdenum cofactor. Plants contain three NifS-like proteins that are localized to the mitochondria (mtNifS), the chloroplast (cpNifS), and the cytosol (ABA3). mtNifS likely provides the S for the formation of biotin and Fe–S cluster assembly for mitochondrial and cytosolic proteins. cpNifS is necessary for Fe–S clusters assembled in the chloroplast, and may also be required for thiamine synthesis. The third NifS-like protein, ABA3, is cytosolic and probably does not participate in Fe–S cluster formation, but rather is required for the sulfuration of molybdenum cofactor. In addition to cysteine desulfurase activity, NifS-like proteins also possess selenocysteine lyase activity that converts selenocysteine into alanine and elemental Se. In contrast to many bacteria, animals, and some green algae that require selenocysteine lyase activity for essential selenoproteins, plants are not known to require Se. However, the selenocysteine lyase activity found in cpNifS may prevent Se toxicity in plants growing in high concentrations of selenate. This review summarizes what is known about NifS-like proteins in plants and discusses other potential roles that still need to be examined.

Keywords: Sulfur; Selenium; Fe–S proteins; Cysteine desulfurase activity

Contents

1. Introduction

It has been nearly 15 years since the discovery of the first NifS protein in the nitrogen-fixing microbe *Azotobacter vinelandii* [1]. Today, NifS-like proteins have been found in all three domains of life, representing a conserved group of proteins with various essential functions. NifS-like proteins have cysteine desulfurase (Cys desulfurase) activity, which catalyzes the conversion of the amino acid cysteine into alanine and elemental sulfur (S) [1]. Cys desulfurase activity provides the S for the formation of a diverse group of cofactors: iron–sulfur (Fe–S) clusters, thiamine, biotin, and molybdenum cofactor. In addition, NifS-like proteins have selenocysteine lyase (SeCys lyase) activity, which converts selenocysteine (SeCys) into alanine and elemental selenium (Se). This reaction plays a role in the formation of essential selenoproteins in organisms that require Se [2].

Eukaryotic NifS-like proteins are nuclear-encoded, and isoforms of the proteins can be found in different intracellular

Abbreviations: S, sulfur; Se, selenium; Cys, cysteine; SeCys, selenocysteine; Cys desulfurase, cysteine desulfurase; SeCys lyase, selenocysteine lyase; Fe–S, iron–sulfur; NA, nicotinic acid; NAD, nicotinamide adenine dinucleotide; cpNifS, chloroplast NifS-like protein; mtNifS, mitochondrial NifS-like protein; SECIS, selenocysteine insertion sequence.

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locations: mitochondria, cytosol, chloroplasts, and occasionally the nucleus. For review of Fe–S clusters, see [3–5]. This range of localization reflects the many processes and cellular components that require the activity of NifS-like proteins. As an example, higher plants contain at least forty Fe–S proteins in multiple cellular compartments that are dependent on NifS-like proteins [4].

Recently the NifS-dependent biogenesis of Fe–S clusters in plants has gained attention [4,5] after initially having been studied only in bacteria, yeast, and mammals. This review describes new insights into the functions of NifS-like proteins in plant Fe–S cluster formation, as well as addresses some remaining questions regarding the other functions of NifS-like proteins in plants. To what extent is the role of NifS-like proteins the same in plants as it is in other organisms, and are there any plant-specific processes? Based on current knowledge, we will discuss why the evolution of land plants probably required the three NifS-like proteins known to exist in plants today. Attention will also be drawn to whether or not the SeCys lyase activity of NifS-like proteins has a function in plants, which is relevant for the debate on whether or not Se is essential for some higher plants.

2. Plant NifS-like proteins are essential for Fe–S cluster maturation

As mentioned, various S-containing cofactors acquire elemental S from the amino acid cysteine via the Cys desulfurase activity of NifS-like proteins, which is reviewed elsewhere [6]. Since Cys is also needed for translation in three cellular compartments, as well as for other S-containing metabolites such as methionine and its derivatives and glutathione, Cys mobilization inside cells must be strictly regulated. Through their Cys desulfurase activity, NifS-like proteins are able to control the formation and intracellular distribution of elemental S.

Fe–S clusters are cofactors for various essential proteins, and are delivered to their appropriate apoproteins by a complex Fe–S cluster biosynthetic machinery [4–7]. NifS-like proteins represent the first step in Fe–S cluster assembly by providing the necessary S for the formation of these essential cofactors. It is still unknown how Fe is delivered to Fe–S clusters; in mitochondria the protein frataxin, which is a Fe chaperone, may have a role in this process [8,9]. Next, the Fe–S cluster must be assembled on a scaffold protein. IscU-like and IscA-like proteins function as scaffold proteins where Fe–S clusters are assembled before the cluster can finally be delivered to its appropriate apoprotein [10]. The general process of Fe–S cluster assembly described above is thought to be the same for the five different types of eukaryotic Fe–S clusters: 2Fe–2S, Rieske-type 2Fe–2S, 3Fe–4S, 4Fe–4S and siroheme 4Fe–4S [4,5].

Fe–S cluster assembly has been extensively studied in bacteria, yeast and mammals. In yeast, Fe–S proteins transfer electrons in the mitochondrial electron transport chain and provide the catalytic activity of aconitase [10]. The deletion of the mitochondrial-localized NifS-like protein is lethal in yeast, which is interesting in view of the fact that yeast are not dependent on mitochondrial electron transport during anaerobic growth. This phenotype can be explained by evidence that essential Fe–S proteins are localized to the cytosol. Thus, *NFS*, the gene encoding a NifS-like protein in yeast mitochondria, is probably essential due to its role in cytosolic, and possibly nuclear, Fe–S assembly [10,11]. Similarly, in human cells, the NifS-like gene *Nfs1* is essential for Fe–S proteins in both the cytosol and mitochondria [12]. However, in this case, differential in-frame start sites ensure that two isoforms of this protein are made which are targeted to the mitochondrion or cytosol, providing evidence for two distinct Fe–S cluster assembly systems in human cells [13]. Additional required components for cytosolic Fe–S cluster assembly have been identified, and termed the cytosolic iron–sulfur cluster assembly machinery [14].

NifS-like proteins can be broadly categorized as belonging to one of two groups based on amino acid analysis of a motif in the active site that contains a conserved Cys [2]. This conserved Cys is the primary acceptor of the sulfane S that results from the cleavage of the substrate cysteine. In type I NifS-like proteins, Cys is exposed in the active site allowing direct delivery of the sulfane S to acceptors, such as scaffold proteins for Fe–S assembly [2,3]. However, this arrangement may make type I NifS-like proteins very oxygen-sensitive. In type II NifS-like proteins, the Cys in the active site is not exposed on the protein surface and is perhaps less oxygen-sensitive. In this case, however, interaction with a SufE protein is required to efficiently release the sulfane S. These SufE proteins serve as intermediate S carriers between NifS and downstream targets [15,16]. Thus, Cys desulfurase activity in group II NifS proteins is substantially lower in the absence of SufE compared to group I. In plants, different SufE proteins exist that reversibly bind to NifS-like proteins and stimulate Cys desulfurase activity [15], as discussed in more detail below.

Some organisms contain more than one NifS-like protein. While the yeast and human genome contain one NifS-like gene, *E. coli* contains three distinct NifS-like proteins: IscS (group I), CsdA (group II), and SufS (group II). IscS seems to have a general housekeeping role, and SufS is thought to function during oxidative stress. Compared to CsdA, SufS has low Cys desulfurase activity in purified form *in vitro* [6]. Whereas IscS and SufS are found in gene clusters with a clear role in the formation of iron–sulfur clusters, CsdA is not in such a cluster and its biological role is not fully clear yet. Deletion of IscS is not lethal in *E. coli*, which is attributed to complementation by the SufS protein [17].

Sequencing of the *Arabidopsis thaliana* genome revealed two NifS-like genes. One NifS-like protein (mtNifS) is localized to mitochondria [18] and the other (cpNifS) is localized to chloroplasts [19,20]. Amino acid sequence analysis reveals that mtNifS (also referred to as NFS1) and cpNifS (also referred to as NFS2) are 18% similar. mtNifS is a group I NifS-like protein and is most similar to bacterial IscS, whereas cpNifS is characterized as a group II NifS-like protein, and is most similar to SufS. Recently, a reduction in mtNifS was shown to decrease the enzyme activities of three aldehyde

oxidases [21]; these Fe–S proteins contribute to the biosynthesis of auxin and abscisic acid in plants. Although it still needs to be confirmed, the mtNifS protein is expected to be critical for the synthesis of Fe–S clusters required by many proteins involved in mitochondrial electron transport. Furthermore, mtNifS may have a role in cytosolic Fe–S assembly [18]. More recently, a third protein designated ABA3 was found to contain a NifS-like domain; ABA3 is required for a late step in abscisic acid biosynthesis, and has Cys desulfurase and SeCys lyase activity *in vitro* [22]. Unlike the other two NifS-like proteins, the cytosolic Cys desulfurase activity of ABA3 is not thought to participate in Fe–S cluster assembly, but rather serves a role in the sulfuration of molybdenum cofactor, as described in more detail below.

The functions of cpNifS were studied by both *in vitro* and *in vivo* approaches. *In vitro*, cpNifS showed both Cys desulfurase and SeCys lyase activity, the latter around 300-fold higher than the former [20]. cpNifS can also mediate *in vitro* reconstitution of an Fe–S cluster in apo-ferredoxin, forming holo-ferredoxin, using Cys and ferrous iron as the sources of S and Fe [23]. Antibody-mediated depletion of cpNifS from a chloroplast extract led to the complete loss of this ferredoxin reconstitution activity. Adding back the corresponding amount of purified recombinant cpNifS restored this activity, indicating cpNifS is the sole protein responsible for providing S for Fe–S clusters in the chloroplast [23].

More recently, the *in vivo* role of cpNifS in Fe–S cluster formation was further studied in *Arabidopsis*. An ethanol-inducible RNAi technique was used to create a conditional mutant; this strategy was chosen because constitutive knockout of cpNifS was lethal [24]. Silencing of cpNifS in mature plants led to a rapid decrease in the levels of chloroplast Fe–S proteins, together comprising all five types of Fe–S clusters. Thus, cpNifS is essential for biosynthesis of all five types of plastid Fe–S clusters, and cpNifS knockout cannot be rescued by other NifS-like plant proteins.

Fe–S proteins in the chloroplast are involved in a diverse range of functions including protein import, chlorophyll metabolism, nitrogen and S reduction, and the photosynthetic electron transport chain. In mitochondria, Fe–S proteins are especially important for respiration. Plants with reduced cpNifS exhibited chlorosis and a disruption in chloroplast structure and function, including photosynthetic electron transport and nitrite reductase activity [24]. Mitochondrial Fe–S proteins and the levels of respiration were not affected by reduced levels of cpNifS, indicating that cpNifS is not responsible for providing mitochondrial Fe–S clusters.

The results described above indicate that the two NifS-like proteins cpNifS and mtNifS fulfill distinct physiological roles suited for their respective environment in plastids and mitochondria [19]. cpNifS and mtNifS probably have different evolutionary origins that fit their intracellular conditions. Although both the chloroplasts and mitochondria have Fe–S proteins involved in electron transport, photosynthesis in the chloroplast generates oxygen while respiration in the mitochondrion consumes oxygen. Thus, the environmental conditions in oxygen-consuming mitochondria and oxygen-producing chloroplasts are different and it is intuitive that these contrasting environmental conditions require two different NifS-like proteins with unique biochemical properties. As mentioned, mtNifS is a group I protein and most similar to IscS, the housekeeping protein in *E. coli* that is very sensitive to oxygen. cpNifS is most similar to bacterial SufS that operates during oxidative stress. Possibly, mtNifS could not function in the chloroplast, because its sensitivity to aerobic conditions would preclude Fe–S cluster development. Future work in plants should demonstrate if cpNifS can indeed tolerate aerobic conditions and oxidative stress better than mtNifS.

Furthermore, since the chloroplast is the predominant site of Cys synthesis, it may be critical to tightly control the Cys desulfurase activity of the chloroplast NifS protein. The presence of a group I NifS-like protein in the chloroplast, with a constitutively high Cys desulfurase activity, could ultimately lead to wasteful Cys cycling. Instead, the Cys desulfurase activity among group II NifS proteins such as cpNifS is relatively low, and can be greatly stimulated by other proteins such as SufE [25]. Work in *E. coli* demonstrates that SufE proteins form a transient complex with NifS proteins, and stimulate transfer of the S released by the NifS protein onto an Fe–S scaffold [15].

Arabidopsis contains three different SufE proteins, termed SufE1, SufE2, and SufE3. SufE1 is nuclear-encoded, but in contrast to the two NifS-like proteins, it shows dual localization to both the mitochondrion and chloroplast, as shown in Fig. 1 [26]. Analogous to *E. coli*, SufE1 and cpNifS were shown to interact and spontaneously form a tetramer *in vitro* [27]. SufE1 is expressed in all tissues and its knockout is lethal. *In vitro*, SufE1 could stimulate both cpNifS and mtNifS, although activation of cpNifS was much more dependent on SufE1 interaction compared to mtNifS [26]. SufE1 can stimulate cpNifS-mediated Cys desulfurase activity 40-fold, and Fe–S cluster formation nearly 20-fold in an *in vitro* reconstitution

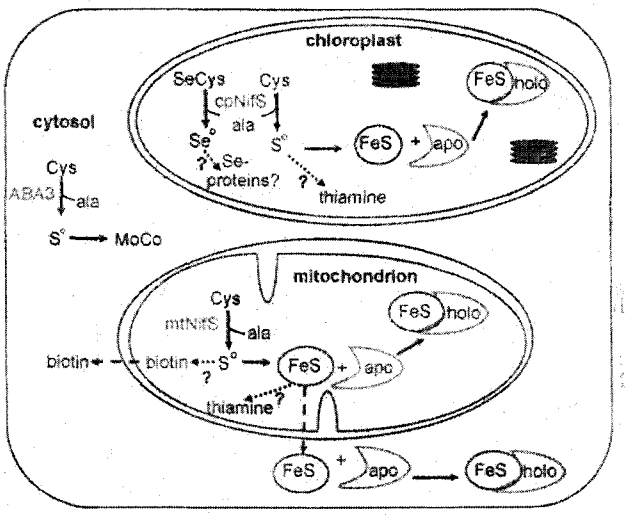

Fig. 1. Known (solid lines) and potential (dotted lines) functions of the three NifS-like proteins in plants (shown in red).

assay [27]. Lethality of SufE1 KO plants can likely be attributed by the mutants' failure to activate cpNifS and form Fe–S clusters.

The two additional chloroplast SufE-like proteins, SufE2 and SufE3, both stimulate cpNifS Cys desulfurase activity at least 40-fold [28]. AtSufE2 is specifically expressed in the pollen in *Arabidopsis* flowers, where it is hypothesized to interact with cpNifS. Unraveling the role of a NifS-like protein in Fe–S cluster development in pollen would be an exciting addition to the field of plant reproductive biology. AtSufE3 is essential, expressed at a relatively low level in all *Arabidopsis* organs, and probably specifically stimulates its own Fe–S cluster formation, as discussed below.

Deletion of IscS in *E. coli* requires the addition of nicotinic acid for growth [29]. The production of quinolinic acid, a precursor of nicotinic acid which is further converted to NAD, requires the activity of the Fe–S protein quinolinate synthase A. In *Arabidopsis*, quinolinate synthase is localized in plastids [30]. Recent evidence has shown that cpNifS is probably essential for NAD synthesis in *Arabidopsis*. A newly identified interacting partner of cpNifS, SufE3 (already mentioned above), contains a quinolinate synthase domain that indeed has quinolinate synthase activity [28]. SufE3 is an essential chloroplast protein, and stimulates cpNifS Cys desulfurase activity up to 70-fold. The quinolinate synthase domain of SufE3 carries an essential, highly oxygen-sensitive 4Fe–4S cluster that SufE3 can reconstitute by means of its own SufE domain, via interaction with cpNifS and using Cys as a S donor [28].

Additional proteins are likely to interact with the SufE1–cpNifS complex, including scaffold proteins. Fe–S proteins are assembled on scaffold proteins before they are transferred to an apoprotein. Potential scaffold proteins in *Arabidopsis* that interact with cpNifS include three Nfu proteins [31,32] and IscA [33]. Fe–S cluster formation in the mitochondria likely involves three Isu scaffold proteins [34], once again demonstrating that two separate Fe–S cluster machineries are required for the mitochondrion and the chloroplast.

3. Other metabolic pathways in plants may require cysteine desulfurase activity

In *E. coli*, Cys desulfurase activity is necessary for the synthesis of other metabolites besides Fe–S clusters, including the vitamins thiamine (B1) and biotin (B8), molybdenum cofactor, and nicotinic acid [6]. The realization that NifS-like proteins fulfill a wide range of roles in *E. coli* begs the question whether the three NifS-like proteins in plants may perform similar functions.

Being autotrophic, plants synthesize their own vitamins. Thiamine (vitamin B1) is a cofactor for enzymes involved in the metabolism of amino acids and carbohydrates. Thiamine possesses a thiazole moiety; the source of this sulfur derives from cysteine, again associating a NifS-like protein with Cys desulfurase activity [35]. In *E. coli*, thiamine biosynthesis requires IscS [36]. This may in analogy suggest that in plants mtNifS is perhaps essential for thiamine formation. Plant thiamine biosynthesis is dependent on Thi1, a protein with two translation start sites, enabling dual localization to the mitochondrion and chloroplast [37]. Currently, it is thought that Thi1 is recruited to the mitochondria where it may prevent DNA damage caused by oxidative stress. Despite this protective role, Thi1 is predominantly localized to the chloroplast where thiamine is perhaps more likely to be made [38]. If this is the case, cpNifS is more likely to be involved in thiamine synthesis than mtNifS. Resolving this issue will be an interesting area of future study.

Biotin (also called vitamin B8 or vitamin H) is an essential cofactor for proteins involved in fatty acid and carbohydrate metabolism [39]. Biotin can only be made in the mitochondria, but can be exported to the cytosol [40]. The last step in biotin production is performed by mitochondrial biotin synthase (Bio2), and involves the transfer of an S atom on dethiobiotin, the precursor of biotin [41]. The S in this reaction comes from Cys, which implies the involvement of a NifS-like protein. Indeed, mtNifS can stimulate biotin production *in vitro* [42]. However, more research is needed to fully understand the exact mechanisms of mtNifS-mediated biotin synthesis.

Sulfuration of molybdenum cofactor is responsible for activating the two enzymes aldehyde oxidase and xanthine dehydrogenase [43]. Research performed by Heidenreich et al. [22] identified a Mo cofactor sulfurase in *Arabidopsis* designated ABA3; this cytosolic protein contains a NifS-like domain with cysteine desulfurase activity that is used for activation of the two MoCo-containing enzymes aldehyde oxidase and xanthine dehydrogenase. Thus, in contrast to mtNifS or cpNifS, the Cys desulfurase activity of ABA3 appears to not have a role in Fe–S assembly, but rather have a specific function in MoCo sulfuration.

4. Plant NifS-like proteins also have selenocysteine lyase activity with unknown function

In addition to having Cys desulfurase activity, NifS-like proteins can also be characterized as SeCys lyases capable of cleaving selenocysteine to form alanine and elemental selenium [2]. A notable difference between Cys desulfurase and SeCys lyase activity in both *E. coli* and *Arabidopsis* is that the conserved Cys residue is very crucial for Cys desulfurase, but not SeCys lyase activity. Also intriguing is that unlike Cys desulfurase activity, SeCys lyase activity is not dependent on SufE activation [23]. Group II NifS-like proteins tend to have a relatively high activity toward SeCys relative to Cys (up to 3000-fold higher), while group I proteins typically have only ~8-fold higher activity toward SeCys than Cys. Due to its low intrinsic CysD activity, purified *Arabidopsis* cpNifS, without SufE, displays nearly 300-fold greater activity toward SeCys than Cys [20].

In organisms that need Se as a micronutrient, NifS-like proteins with SeCys lyase activity provide the elemental Se for the formation of specific selenoproteins containing SeCys. SeCys, dubbed the 21st amino acid, is encoded by a UGA opal codon in mRNAs encoding selenoproteins [44]. At least 25

selenoproteins are predicted in humans, many of which are active in redox reactions, such as the enzyme glutathione peroxidase [45]. Glutathione peroxidases play a role in the scavenging of free radicals; a human glutathione peroxidase with a structural role in the maturation of sperm has also been proposed [46]. Plant glutathione peroxidases contain Cys at the active site instead of SeCys, and indeed no selenoproteins have been found so far in higher plants. Thus, while Se is an essential micronutrient for many animals and bacteria, as well as for the green algae *Chlamydomonas reinhardtii* [47] and *Emiliana huxleyi* [48], to date there is no direct evidence that Se is essential for higher plants. Moreover, the typical components required for the insertion of SeCys during translation are absent in plants. Coming back to NifS-like proteins, there is therefore no evidence that cpNifS or other plant NifS-like proteins have a role in essential selenoprotein synthesis.

It is worth mentioning the limited, yet exciting evidence from outside the plant kingdom, that a cpNifS homologue may be involved in selenoprotein syntheses. *Plasmodium falciparum*, the causative agent of malaria, and other apicomplexan parasites harbor apicoplasts [49]. These unique structures are non-photosynthetic plastids probably derived from cyanobacteria during a series of endosymbiotic events. In addition to a mitochondrial NifS-like protein, *Plasmodium* spp. contain a group II NifS-like protein (acNifS) probably localized to the plastid, that is homologous to cpNifS. In the future it will be interesting to determine how acNifS functions in these apicomplexans, and if they all have a similar role to the NifS-like protein in chloroplasts. Obviously, these heterotrophic parasites do not require Fe–S proteins for photosynthetic electron transport. Could these plastidic proteins provide the SeCys lyase activity necessary for selenoprotein synthesis? Keeping in mind that these organisms contain selenoproteins and that acNifS is similar to SufS and cpNifS, it is quite likely that acNifS functions as a SeCys lyase. Regardless of its function, targeting the *Plasmodium* spp. plastid NifS-like gene product may be a viable strategy to control malaria [49,50].

If cpNifS' SeCys lyase activity has a function in plants, other processes besides a role in selenoprotein synthesis should be considered. Is it feasible that SeCys lyase activity may function to prevent Se toxicity in plants? At higher levels Se is toxic to most plants, as it is to other organisms. This toxicity stems from nonspecific incorporation of SeCys and SeMet into proteins, replacing Cys and Met [51]. Se toxicity in plants manifests itself by stunted growth and chlorosis of young leaves. Overexpression of cpNifS in *Arabidopsis* increased tolerance to and accumulation of toxic Se, but did not bestow any additional benefits during oxidative stress, low light, or Fe and S starvation [52]. It is speculated that overexpression of cpNifS diverted SeCys away from nonspecific incorporation into protein, thereby allowing the plants to tolerate and accumulate more Se. Indeed, less Se was incorporated into protein in cpNifS-overexpressing transgenics [52]. These observations create the possibility that SeCys lyase activity may have a function in preventing Se toxicity in plants growing on elevated levels of Se. Considering that, on a global scale, only a small fraction of soils is seleniferous, it is worth pondering what selective pressures have preserved SeCys lyase activity in the cpNifS protein. Alternatively, it is possible that SeCys lyase activity is an inevitable side-effect of having Cys desulfurase activity, since Cys and SeCys are very similar. It cannot be excluded that new evidence will reveal a role for plant NifS-like proteins in plant Se metabolism.

5. Concluding remarks

Exciting insights into the function of plant NifS-like proteins have begun to emerge since the start of the genomics era. As depicted in Fig. 1, NifS-like proteins in plants are localized to the mitochondria (mtNifS), the chloroplast (cpNifS), and the cytosol (ABA3). All three NifS-like proteins have Cys desulfurase and SeCys lyase activity, the latter of which may have a protective role in preventing toxic Se incorporation into protein. Studies so far have clearly shown the importance of cpNifS for chloroplast Fe–S cluster biosynthesis, and there is evidence that mtNifS is needed for Fe–S cluster formation in mitochondria and cytosol. Besides Fe–S clusters, plant NifS-like proteins also are needed for the synthesis of other cofactors. For example, cpNifS is needed for NAD synthesis and ABA3 for cytosolic MoCo sulfuration. It is also likely that mtNifS is needed for biotin synthesis, and either cpNifS or mtNifS may provide the S for thiamine. The contrasting conditions of the mitochondria and chloroplast probably necessitate two distinct NifS-like proteins for the maturation of Fe–S clusters in these two organelles, each with different properties. Future work may focus on identifying regulatory mechanisms for the NifS–SufE interactions, on demonstrating whether or not mtNifS is indeed required for cytosolic Fe–S cluster assembly, and on the possible functions of the SeCys lyase activity of NifS-like proteins in plants.

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Overexpression of AtCpNifS Enhances Selenium Tolerance and Accumulation in Arabidopsis[1]

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Selenium (Se) is an essential element for many organisms but is toxic at higher levels. CpNifS is a chloroplastic NifS-like protein in Arabidopsis (*Arabidopsis thaliana*) that can catalyze the conversion of cysteine into alanine and elemental sulfur (S^0) and of selenocysteine into alanine and elemental Se (Se^0). We overexpressed CpNifS to investigate the effects on Se metabolism in plants. CpNifS overexpression significantly enhanced selenate tolerance (1.9-fold) and Se accumulation (2.2-fold). CpNifS overexpressors showed significantly reduced Se incorporation into protein, which may explain their higher Se tolerance. Also, sulfur accumulation was enhanced by approximately 30% in CpNifS overexpressors, both on media with and without selenate. Root transcriptome changes in response to selenate mimicked the effects observed under sulfur starvation. There were only a few transcriptome differences between CpNifS-overexpressing plants and wild type, besides the 25- to 40-fold increase in CpNifS levels. Judged from x-ray analysis of near edge spectrum, both CpNifS overexpressors and wild type accumulated mostly selenate (Se^{VI}). In conclusion, overexpression of this plant NifS-like protein had a pronounced effect on plant Se metabolism. The observed enhanced Se accumulation and tolerance of CpNifS overexpressors show promise for use in phytoremediation.

Selenium (Se) is an essential micronutrient for animals and bacteria but becomes toxic at higher concentrations. Accumulation of Se in the environment has been reported to be a cause of toxicity in aquatic organisms (Ohlendorf et al., 1988; Hamilton, 2004), livestock (Fessler et al., 2003), and humans (Hira et al., 2004). Selenate (SeO_4^{2-}) is the most common soluble form of Se found in terrestrial systems. Seleniferous soils (>2 ppm Se) commonly found in the western United States arise from the weathering of Se-rich shale rock.

Se is essential for mammals and bacteria, where selenocysteine (SeCys) is an essential component for the formation of selenoenzymes such as glutathione peroxidase, a free radical scavenger (Stadtman, 1990). A diet enriched with Se can reduce the risk of cancer in humans (Ip et al., 2002). Thus, plants enriched with anticarcinogenic selenocompounds have enhanced nutritional value (Ellis et al., 2004). Whether or not Se is essential for higher plants themselves remains unproven. However, the green alga *Chlamydomonas reinhardtii*, which shares a common ancestor with land plants, was shown to contain selenoproteins. Labeling of *C. reinhardtii* cells with ^{75}Se revealed four Se proteins, one of which is a glutathione peroxidase, thought to ameliorate oxidative stress (Novoselov et al., 2002).

Although certain plants tolerate and (hyper)accumulate Se to high concentrations (up to 0.5% of dry weight), generally Se uptake into plant tissue is thought to be inadvertent and potentially toxic (Anderson, 1993). The toxicity of selenate is thought to be due to its chemical similarity to sulfate, and, as such, proteins involved in sulfate transport and sulfur (S) metabolism can act on analogous Se compounds (Terry et al., 2000). When selenate is assimilated via the sulfate assimilation pathway, Se can be incorporated into the S amino acids Cys and Met, leading to the formation of SeCys and selenomethionine. The nonspecific incorporation of SeCys into proteins causes toxicity (Stadtman, 1990).

Attenuating the toxic effects of Se is of concern for public health and agriculture. Plants with enhanced Se tolerance and accumulation may potentially be used for environmental cleanup (Bañuelos et al., 2005). Several strategies have been used successfully to breed plants with enhanced Se tolerance and accumulation. Nonspecific incorporation of SeCys into proteins can

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be prevented by methylation of SeCys into nonprotein selenoamino acids via the enzyme SeCys methyl transferase (SMT; Neuhierl and Böck, 1996). Overexpression of this enzyme from the Se hyperaccumulator *Astragalus bisulcatus* in Arabidopsis (*Arabidopsis thaliana*) or Indian mustard (*Brassica juncea*) enhanced Se tolerance and accumulation (Ellis et al., 2004; LeDuc et al., 2004). Methylation of selenomethionine and SeCys can lead to the formation of volatile selenocompounds, another potential detoxification mechanism (Terry et al., 2000). Overexpression in Indian mustard of cystathionine-γ-synthase, the first enzyme in the conversion of SeCys to volatile Se, led to increased levels of dimethylselenide production and increased Se tolerance (Van Huysen et al., 2003). Overexpression of the key enzyme of the sulfate assimilation pathway, ATP sulfurylase, led to enhanced selenate reduction, Se accumulation, and Se tolerance (Pilon-Smits et al., 1999). In another study, barley (*Hordeum vulgare*) plants overexpressing thioredoxin *h* were capable of tolerating and accumulating more Se when grown on selenite (Kim et al., 2003) perhaps due to enhanced reduction of selenite to elemental Se (Se^0). In another approach to prevent Se incorporation into protein, a mouse gene expressing a SeCys lyase (SL) was introduced into Arabidopsis and Indian mustard (Garifullina et al., 2003; Pilon et al., 2003). The enzyme proved capable of reducing the amount of Se in protein, probably by cleaving SeCys into alanine and Se^0. The plants accumulated more Se when the gene was overexpressed in either the cytosol or the chloroplast, but tolerance to Se was enhanced only when expressed in the cytosol.

Recently, a chloroplastic protein, CpNifS, was discovered in Arabidopsis that has both Cys desulfurase and SL activity (Pilon-Smits et al., 2002), and thus potentially affects both S and Se metabolism. CpNifS can provide the necessary S to form iron (Fe)-S clusters for ferredoxin by cleaving Cys into elemental S and alanine (Ye et al., 2004; Abdel-Ghany et al., 2005), and possibly has a role in providing the elemental S for other S metabolites such as thiamine, in analogy with bacterial systems (Mihara and Esaki, 2002). However, the activity of this enzyme toward SeCys is almost 300 times higher than toward Cys (Pilon-Smits et al., 2002), suggesting CpNifS may affect plant Se metabolism as well. Since CpNifS converts SeCys into alanine and Se^0, this enzyme may prevent Se incorporation into protein, which should protect plants from Se toxicity.

In this study, we overexpressed CpNifS in Arabidopsis to study the effects on Se and S metabolism. The effects on Se metabolism were explored by determining the ability of the transgenic plants to tolerate and accumulate Se, and the metabolic fate of the accumulated Se. Microarray analysis was performed to more fully understand how overexpression of CpNifS may affect Se tolerance and accumulation, and to investigate the transcriptome changes that occur when plants are grown on selenate.

RESULTS

Generation and Characterization of Transgenic Lines

The CpNifS cDNA, including its plastid targeting sequence (Fig. 1), was constitutively expressed in Arabidopsis. The CpNifS protein expression level in the transgenic plant lines obtained was tested by immunoblotting using antibodies raised against purified CpNifS. A protein (43 kD) of the expected size for the mature protein after cleavage of the transit sequence was present at much higher levels in shoot and root tissues of the CpNifS lines (Fig. 2A) compared to wild type. The degree of overexpression varied among the CpNifS transgenic lines. In general, CpNifS expression was higher in shoot than root tissue in the transgenics, despite the use of the constitutive promoter (Fig. 2A). Transgenic lines CpNifS38 and CpNifS55 were chosen for further analysis. Judged from digital imaging, these two lines contained about 23-fold higher CpNifS protein concentration compared to wild type in the shoots. In root material, CpNifS levels were roughly 8-fold higher in the transgenics. To verify the localization of CpNifS in the chloroplasts, a western-blot analysis was performed on isolated chloroplasts from wild type, CpNifS38 and CpNifS55 (Fig. 2, B and C). The level of CpNifS protein in all plant types was slightly lower in the chloroplasts compared to homogenate, when compared on a chlorophyll basis. Since CpNifS was shown earlier to be chloroplastic (Pilon-Smits et al., 2002), some of the stromal proteins may be lost due to leakage in this procedure. The fraction of CpNifS in chloroplasts was similar in CpNifS55 compared to wild type, but somewhat lower in CpNifS38. Thus, it appears that the majority of the overproduced CpNifS was indeed targeted to the chloroplast, but it cannot be excluded that some of the protein was mistargeted, especially in CpNifS38.

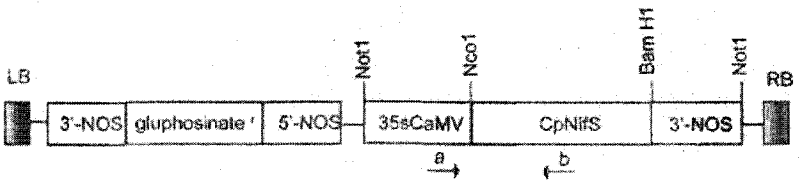

Figure 1. AtCpNifS construct used to transform Arabidopsis. Shown are the forward (a) and reverse (b) primers that were used to identify transgenic lines. 3′-NOS and 5′-NOS, Nopaline synthase terminator and promoter, respectively. LB and RB, Left border and right border of T-DNA, respectively.

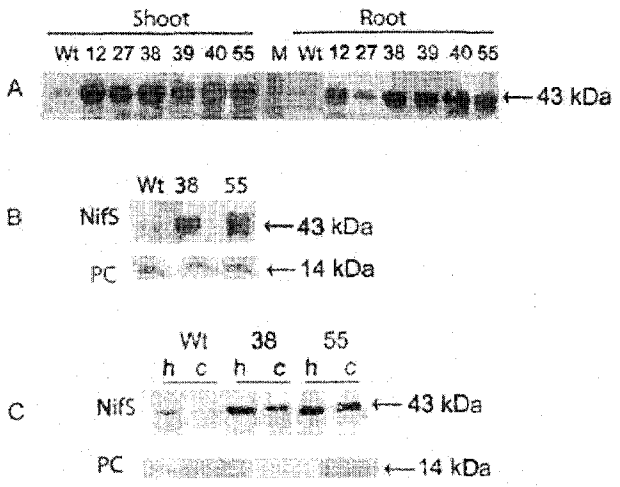

Figure 2. A, Immunoblot showing CpNifS overexpression in roots and shoots of six different homozygous CpNifS lines and wild type (Wt). Twenty micrograms of total protein was loaded per lane. Lines 38 and 55 were selected for further study. B and C, Immunoblot analysis of CpNifS and plastocyanin (PC) in homogenate (h) and isolated chloroplasts (c) from wild type and CpNifS-overexpressing lines 38 and 55. Three micrograms of chlorophyll was loaded per lane. Plastocyanin was visualized as a control for equal loading.

Effects of CpNifS Overexpression on Se and S Metabolism

Since CpNifS can convert SeCys into Se^0 and alanine, thereby potentially preventing the toxic process of nonspecific incorporation of Se into protein, we wanted to test whether CpNifS overexpression affects Se tolerance. The ability of the CpNifS overexpressors to tolerate selenate was investigated by measuring root growth on media with or without 40 μM selenate. The transgenics CpNifS38 and CpNifS55 had 1.7- to 2-fold longer roots after 10 d of treatment on selenate compared to wild type ($P < 0.05$; Fig. 3). Interestingly, the CpNifS transgenics did not show enhanced selenite tolerance (data not shown). There were also no differences in root length between wild-type and transgenic plants grown on control (Murashige and Skoog [MS]) media (Fig. 3) or on media supplied with cadmium or chromate (data not shown), indicating that CpNifS overexpression enhances selenate tolerance and the observed effect is selenate specific.

To determine if CpNifS overexpression alters Se accumulation, seedlings of wild type, CpNifS38, and CpNifS55 were grown on medium with 40 μM selenate. Transgenic plants accumulated more Se in shoot and root tissues than wild type ($P < 0.05$; Fig. 4a); the Se concentration in shoots was 2- to 3-fold higher in transgenics compared to wild type. The Se concentration in the shoots exceeded that in the roots for all three genotypes, agreeing with previous results that plants readily translocate Se to the shoot when supplied with selenate (de Souza et al., 1998; Pilon et al., 2003; LeDuc et al., 2004). Interestingly, the translocation of Se (the ratio of shoot Se to root Se) was more than 50% higher in CpNifS38 and CpNifS55 compared to wild type ($P < 0.05$; Fig. 4a).

The observation that CpNifS overexpressors are more tolerant to Se, yet also accumulate more Se, suggests that their tolerance mechanism does not involve Se exclusion by the plant. Rather, overexpressors may have altered Se metabolism, resulting in accumulation of less toxic Se forms, or altered Se compartmentation. We hypothesized that these transgenics were better able to tolerate selenate because they are capable of shuttling Se away from protein incorporation by breaking down SeCys into Se^0 and alanine. To test this hypothesis, the amount of Se in protein was determined. Indeed, the CpNifS transgenics incorporated approximately a third less Se in protein than the wild-type plants ($P < 0.05$; Fig. 5). Since nonspecific incorporation of Se into proteins is toxic, the capacity of the CpNifS overexpressors to prevent this toxic process likely caused the observed increased tolerance to Se.

Micro x-ray fluorescence (μXRF) mapping and micro x-ray absorption spectroscopy (μXAS) were used to determine and compare the Se speciation in the leaves of wild type and the highest CpNifS overexpressor, CpNifS55. In addition to Se located inside cells, both plant types clearly show abundant levels of Se in their vascular tissue (Fig. 6, left). Judged from x-ray analysis of near edge spectra (XANES), there were no major differences between the transgenic and wild type with respect to the oxidation state of Se (Fig. 6, right). Both lines contained predominantly selenate with a small fraction of more reduced Se, as indicated by the shoulder at 12,660 eV. This possibly was an organic Se species (Pilon-Smits et al., 1999), Se^0 or selenite. Judged from linear least-squares fitting to the three references, on average the fraction of Se present as selenate was 70% for wild type and 66% for CpNifS55. The chemical form of the remaining, more reduced Se cannot be determined with certainty from this limited number of references, but the fraction of more reduced Se may be a bit higher for CpNifS than for wild type, judged from the slightly more pronounced shoulder at 12,663 eV (Fig. 6) and the

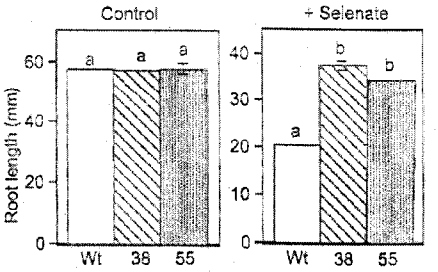

Figure 3. Se tolerance as determined by measuring the root length of wild-type (Wt), CpNifS38, and CpNifS55 seedlings grown for 10 d on MS medium with and without 40 μM selenate. Shown are the mean ($n = 25$) and SE of the mean. Lowercase letters above bars denote significant differences ($P < 0.05$).

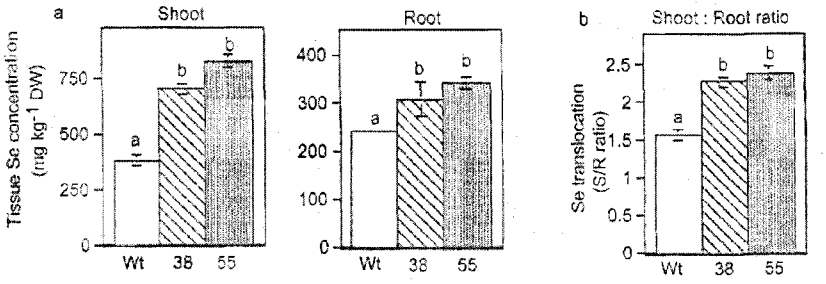

Figure 4. A, Shoot and root Se concentration in wild-type (Wt) and CpNifS-overexpressing (38 and 55) plants grown on MS medium containing 40 μM selenate for 20 d. Shown are the mean and SE of the mean for the shoots and roots ($n = 5$ pooled samples from 20 seedlings each). B, Se translocation as determined by calculating the shoot to root ratio of all three lines. Lowercase letters above bars denote significant differences ($P < 0.05$).

corresponding least-squares fitting analysis described above.

CpNifS can act on both Cys and SeCys, and as such its overexpression may affect both S and Se metabolism. Accumulation of S was therefore also analyzed. Under control conditions, CpNifS38 and CpNifS55 had higher levels of S in shoots, but not in roots, compared to wild type ($P < 0.05$; Fig. 7a). Additionally, CpNifS overexpression led to an increase in tissue S concentration in both shoots and roots compared to wild type when grown on selenate (Fig. 7b), suggesting a stimulating effect of CpNifS overexpression on S uptake. Selenate treatment also enhanced S uptake; compared to accumulation of S on control media, more S was accumulated in the shoots of all three genotypes grown on selenate.

Microarray Analysis

GeneChip arrays (Affymetrix) were used to identify differences in Se-related transcriptome responses between CpNifS overexpressors and wild type, and to determine how growth on selenate changes the transcriptome of Arabidopsis. Transcriptome profiling was performed on roots of wild-type, CpNifS38, and CpNifS55 plants that were grown from seed on half-strength MS agar media with and without 40 μM selenate for 10 d.

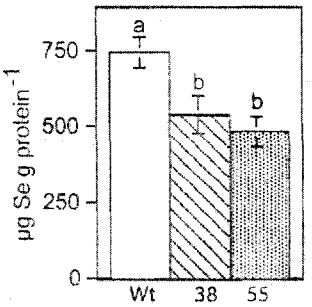

Figure 5. Se incorporation into protein in shoot tissue of wild-type (Wt) and CpNifS-overexpressing (38 and 55) seedlings grown for 14 d on 20 μM selenate. Shown are the mean and SE of the mean ($n = 5$ pooled samples from 50 seedlings each). Lowercase letters above bars denote significant differences ($P < 0.05$).

We chose to focus on root tissue because we found clear differences between CpNifS plants and wild type with respect to root growth. Also, the transgenics differed in Se and S uptake and translocation, and we hypothesized that there would be differences in the expression of root sulfate transporters involved in sulfate/selenate uptake into the root symplast and the root xylem.

The microarray experiment confirmed the overexpression of CpNifS in the transgenic plants. Line CpNifS38 showed a 27-fold increase in CpNifS expression, and CpNifS55 had 40-fold higher CpNifS mRNA levels compared to wild type on control medium (Table I). These data confirm the overexpression observed at the protein level (Fig. 2). CpNifS transcription levels were not affected by the selenate treatment in wild type, i.e. the endogenous transcript level of CpNifS was not affected by selenate (Table I). However, preliminary results (H. Ye, M. Pilon, and E. Pilon-Smits, unpublished data) indicate that CpNifS is up-regulated by selenate at the protein level.

In both the wild type and CpNifS overexpressors, selenate treatment had a pronounced effect on the root transcript level of many genes involved in sulfate uptake and assimilation (Table II). Sulfate transporters were generally up-regulated when grown on Se, particularly SULTR1;1, SULTR2;1, and SULTR4;2. SULTR1;2 and SULTR4;1 were also up-regulated by Se. In the primary S-assimilation pathway, several ATP sulfurylase genes (APS1 and 3), APS reductase genes (APR1, 2, and 3), *O*-acetylserine (thiol) lyase (Bsas4;2), Ser acetyltransferase (Serat3;1), and a putative cystathionine γ-lyase (At1g64660) were up-regulated by selenate in all plant types. S-related genes that were down-regulated on selenate include APS2 and 4, an *O*-acetylserine (thiol) lyase (Bsas1;4), and γ-glutamylcysteine synthetase (GSH1). A homocysteine *S*-methyltransferase (HMT-3), homologous to the SMT of the Se hyperaccumulator *A. bisulcatus* (Neuhierl et al., 1999), was differentially regulated by selenate in wild type compared to the transgenics. While in wild type the HMT-3 transcript levels were not affected by Se, they decreased 2- to 3-fold in CpNifS plants. Apart from HMT3, a few other genes were differentially regulated by Se in the CpNifS roots compared to wild type (Table I). Other potentially Se-related genes that were regulated by selenate are two homologs of Se-binding proteins (At3g23800

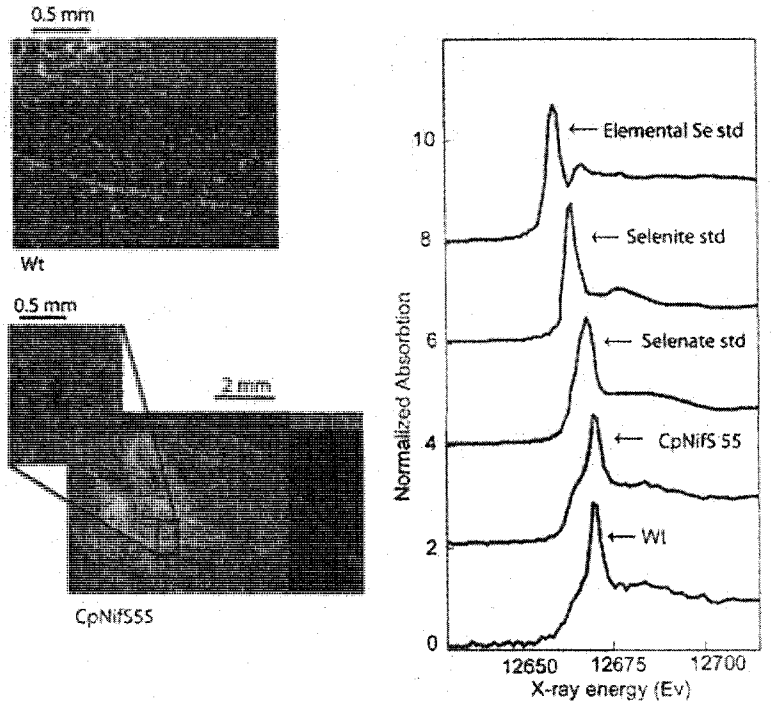

Figure 6. Left, μXAS analysis showing spatial Se distribution in leaves of wild type and CpNifS55. Right, Representative XANES spectra obtained from the leaves shown, in comparison with Se standards selenate, selenite, and elemental (gray) Se.

and At4g14030/At4g14040; Table II). Both were regulated approximately 2.5-fold by Se treatment in all plant types. However, At3g23800 was down-regulated while At4g14030/At4g14040 was up-regulated.

DISCUSSION

Overexpression of CpNifS, a NifS-like chloroplast protein with SL activity, resulted in 2-fold enhanced tolerance to selenate and 2- to 3-fold higher Se accumulation. Because CpNifS mediates the breakdown of SeCys into alanine and Se^0, we hypothesized that the transgenic plants increased selenate tolerance because of decreased SeCys incorporation into protein. Indeed, the transgenics showed significantly reduced incorporation of Se into protein. Therefore, this NifS-like endogenous plant protein clearly affects plant Se metabolism when overexpressed.

The CpNifS transgenics showed enhanced tolerance to selenate but not to selenite. This may be because the conversion from selenate to selenite is a very slow and rate-limiting step in Se assimilation, especially in Arabidopsis (de Souza et al., 1998; Pilon-Smits et al., 1999; Ellis et al., 2004). The slow production of SeCys in selenate-supplied plants may enable the CpNifS protein to keep up with its detoxification, preventing it from being incorporated into protein. Selenite-supplied plants were shown to quickly convert all Se into organic Se species (de Souza et al., 1998), and this flood of SeCys may have saturated the detoxification capacity of CpNifS in the transgenics.

In a previous study, a mouse SL was expressed in Arabidopsis (Pilon et al., 2003). When this mouse SL was expressed in the cytosol, the plants showed enhanced Se tolerance, but when expressed in the plastids the plants became less tolerant to Se. It was speculated that the produced Se^0 may replace elemental S and interfere with Fe-S cluster assembly in the chloroplast. It is intriguing that overexpression of the mouse SL in the chloroplast decreased Se tolerance while overexpression of this endogenous Arabidopsis SL enhanced Se tolerance. This could be due to different SeCys/Cys substrate specificities of both enzymes. In vitro CpNifS showed a 300-fold higher activity toward SeCys compared to Cys, while the mouse SL had 5,000-fold higher activity toward the Se substrate (Esaki et al., 1982; Pilon-Smits et al., 2002). Also, there may be regulatory mechanisms in the chloroplast that modulate CpNifS (but not mouse SL) activity toward both substrates. These may allow CpNifS to protect against both Se incorporation into proteins and Fe-Se clusters. Finally, it cannot be excluded that not all CpNifS was targeted to the plastids in the transgenics, but that a small fraction was present in the cytosol, leading to a phenotype similar to that found when the mouse SL was expressed in the cytosol.

Another difference between the transgenics overexpressing the mouse SL in the chloroplast (cpSL) and the CpNifS transgenics described here was that the mouse cpSL plants did not show any difference in Se accumulation from selenate, while the CpNifS plants did. Again, this could be due to different SeCys/Cys

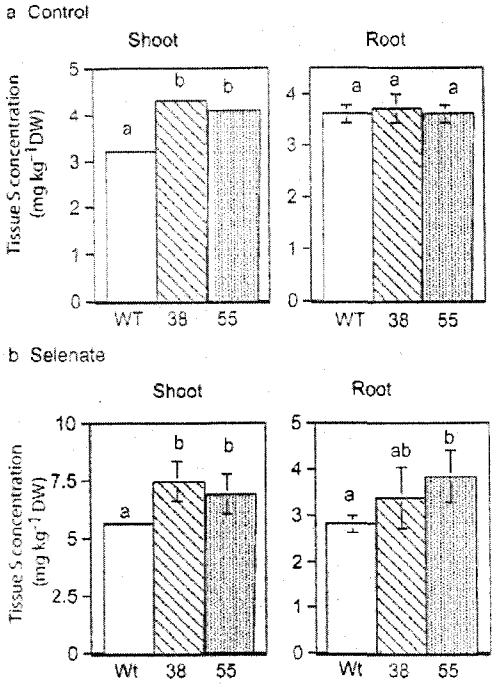

Figure 7. Shoot and root S concentrations in wild-type (Wt) and CpNifS-overexpressing (38 and 55) seedlings grown on medium with and without 40 μM selenate for 20 d. Shown are the means and SE of the mean (n = 5 pooled samples from 20 seedlings each). Lowercase letters above bars denote significant differences ($P < 0.05$).

substrate specificities of both enzymes. The mouse SL has negligible activity toward Cys, in contrast to CpNifS (Esaki et al., 1982; Pilon-Smits et al., 2002). Perhaps the increased uptake of selenate and sulfate are correlated with the breakdown of Cys.

CpNifS diverts Se away from protein incorporation, and it was hypothesized that this mechanism may allow transgenic plants to accumulate a different form of Se (e.g. Se^0) compared to wild type. Indeed, there appeared to be a slightly higher fraction of more reduced Se in the transgenics judged from μXAS analysis. However, the majority of Se was present as Se^{VI} in both wild type and CpNifS transgenics. It is possible that there was substantial accumulation of Se^0 in the plastids, but this was masked by the abundance of selenate in other cellular compartments. In addition, the elevated S levels in the CpNifS transgenics may have contributed to their enhanced selenate tolerance, as they would allow S in cells to compete more successfully with Se for incorporation into S compounds.

In both wild type and CpNifS55, Se appeared to accumulate predominantly in the periphery of the cells and in the vascular tissue. The resolution does not enable us to discern between the cell wall, cytosol, or plastids, but it does not appear that Se was accumulated in the vacuoles. The high Se concentration in the vascular tissue may indicate that uptake into the shoot symplast was a limiting factor for Se accumulation in the leaf under these conditions.

Overexpression of CpNifS resulted in enhanced accumulation of both Se and S in selenate-treated plants; S was also accumulated more in shoots of CpNifS transgenics than in wild type under control conditions. This suggests that the breakdown of (Se)-Cys enhances selenate and sulfate uptake. The enhanced Se accumulation in the CpNifS transgenics does not appear to be caused by more pronounced up-regulation of sulfate transporters at the transcriptional level, at least not in the root, since the levels of up-regulation were similar to wild type. Still, it is possible that S and Se fluxes through the plant differed in wild type and CpNifS transgenics via regulation at the protein level, or via differences in shoot transcription levels or transcriptional differences in certain cell types.

In all plant types, S levels were higher in selenate-treated plants compared to control plants, which can be explained by the observed up-regulation of various sulfate transporters. A similar up-regulation of sulfate transporters as well as of genes involved in sulfate assimilation (Leustek et al., 1994; Gutierrez-Marcos et al., 1996; Setya et al., 1996) and Cys synthesis (Hatzfeld et al., 2000; Kawashima et al., 2005) was observed in earlier studies where plants were subjected to S deficiency (Takahashi et al., 1997, 2000; Yoshimoto et al., 2002; Maruyama-Nakashita et al., 2003, 2005; Kataoka et al., 2004). Thus, selenate may be perceived by the plant as S deficiency, or even cause deficiency of certain S-containing metabolites. As mentioned above, the higher S levels in the CpNifS transgenics compared to wild type on Se may provide the plants with an additional mechanism to alleviate stress.

The discovery that an endogenous NifS-like protein with SL activity can affect plant Se metabolism is intriguing, in view of the current thought that Se is not an essential micronutrient for higher plants, as opposed to the green alga Chlamydomonas (Novoselov et al., 2002). The SL activity of CpNifS may be an evolutionary relic of essential Se metabolism or an unavoidable side reaction of a protein with Cys desulfurase function. It is feasible that in some species CpNifS has a function in Se metabolism by preventing nonspecific Se incorporation into protein, rather than providing Se^0 for essential selenoprotein synthesis like it does in mammals and bacteria (Stadtman, 1990; Mihara and Esaki, 2002). However, this is probably not common as there are not many natural areas in the world where plants experience Se toxicity. Incidentally, the preference of CpNifS for SeCys over Cys may not be as pronounced in planta as it is in vitro (300-fold). Recently, a SufE-like chloroplast protein was shown to stimulate the Cys desulfurase activity of CpNifS 40-fold in vitro and to increase its affinity toward Cys 2-fold (H. Ye, S. Abdel-Ghany, E. Pilon-Smits, and M. Pilon, unpublished data). This protein likely regulates CpNifS activities toward its Se and S substrates in vivo.

Table I. *Transcriptome differences between CpNifS overexpressors and wild-type plants*

Wild-type (ecotype Ws) and CpNifS-overexpressing (NifS55 and NifS38) Arabidopsis plants were grown on control medium (MS) or with 40 μM selenate (Se) for 10 d, and transcriptomes of root RNA were analyzed using Affymetrix GeneChip arrays as described in "Materials and Methods." Two sets of experiments (set 1 and set 2) were carried out separately to evaluate the reproducibility of the results. Genes showing more than 2-fold differences between the wild type and CpNifS overexpressors either on MS or Se conditions were selected by comparing the normalized signals and calculating their ratios, as shown in the NifS55/Ws and Nif38/Ws columns. The significance of the difference is represented by superscript letters, which describe the up and down effects of NifS overexpression on gene expression by fold change differences: a, >5.00; b, >2.00; c, >1.33; d, <0.75; e, <0.50. Regulation by Se (Se/MS ratio) is indicated using the same superscript letters. The absolute calls of each transcript (P, present; M, marginal; A, absent) are indicated on the right-hand side of the normalized values. The relative ratios are calculated for the pairs having "present" call at least in one sample.

Affy Code	AGI Locus	Annotation	Set 1								Set 2							
			Ws			NifS55			NifS55/Ws		Ws			NifS38			NifS38/Ws	
			Se	MS	Se/MS	Se	MS	Se/MS	Se	MS	Se	MS	Se/MS	Se	MS	Se/MS	Se	MS
261744_at	AT1G08490	NifS	1.16 P	1.03 P	1.12	41.32 P	47.44 P	0.87	35.71[a]	46.10[a]	0.69 P	0.75 P	1.19	24.52 P	20.01 P	1.23	27.46[a]	26.75[a]
259161_at	AT3G01500	Carbonic anhydrase 1	0.33 A	9.36 P	0.04[e]	2.03 A	0.42 A			0.04[e]	0.25 A	8.47 P	0.03[e]	0.73 A	3.30 A			0.39[e]
258897_at	AT3G05730	Expressed protein	0.39 A	1.08 P	0.36[e]	1.33 P	0.41 P	3.22[b]	3.41[b]	0.38[e]	0.49 A	1.07 P	0.46[e]	1.16 P	1.75 P	0.66[d]	2.36[b]	1.64[c]
252537_at	AT3G45710	Proton-dependent oligopeptide transporter family	0.25 P	1.16 P	0.22[e]	0.84 P	1.49 P	0.56[d]	3.35[b]	1.29	0.17 P	0.82 P	0.21[e]	0.45 P	0.66 P	0.68[d]	2.57[b]	0.81
260535_at	AT2G43390	Expressed protein	0.61 P	1.13 P	0.54[d]	1.56 P	1.44 P	1.09	2.55	1.28	0.71 A	1.42 P	0.50[d]	1.30 P	1.75 P	0.86	2.10[b]	1.23
251974_at	AT3G53200	Myb family transcription factor (MYB27)	0.64 M	1.33 P	0.48[e]	1.46 P	2.67 P	0.55[d]	2.27	2.00[b]	0.84 P	1.13 P	0.74[d]	1.78 P	1.62 P	1.10	2.11[b]	1.43[c]
264263_at	AT1G09155	SKP1 interacting partner 3-related	0.51 A	0.69 P	0.74[d]	1.10 P	1.66 P	0.66[d]	2.15	2.39[b]	0.46 A	1.58 P	0.29[e]	1.19 P	0.90 P	1.33	2.59[b]	0.57[d]
255054_s_at	AT4G23560	Glycosyl hydrolase family 9 protein *similar to cellulase*	1.44 P	0.80 P	1.80[c]	0.73 P	2.41 P	0.30[e]	0.51[d]	3.02[b]	0.45 A	0.82 M		0.99 P	1.73 P	0.57[d]	2.21[b]	2.11[b]
246340_s_at	AT3G44860	S-Adenosylmethionine: carboxyl methyltransferase family	15.18 P	0.65 A	23.50[a]	5.93 P	1.33 P	4.45[b]	0.39[e]	2.06[b]	10.71 P	0.35 A	30.34[a]	7.94 P	1.18 P	6.73[a]	0.74[d]	3.33[b]
247905_at	AT5G57400	Hypothetical protein	0.73 P	1.00 A	0.72[d]	0.67 P	2.02 P	0.43[e]	1.20	2.01[b]	1.07 A	0.76 M		1.15 A	1.70 P	0.68[d]		2.23[b]
265400_at	AT2G10940	Protease inhibitor/seed storage/LTP family	0.30 A	2.86 P	0.10[e]	0.33 A	0.77 A			0.27[e]	0.64 A	3.56 P	0.18[e]	0.91 A	1.63 A			0.46[e]
249390_at	AT5G39740	60S ribosomal protein L5	1.35 A	1.09 P	1.23	1.09 A	0.30 A	3.66[b]		0.27[e]	0.86 A	1.36 P	0.63[d]	1.04 A	0.29 A			0.21[e]
263369_at	AT2G20480	Expressed protein	1.38 P	1.32 P	1.05	1.05 P	0.45 A	2.32[b]	0.76	0.34[e]	0.89 P	1.07 P	0.83	0.44 A	0.40 A		0.49[e]	0.37[e]
261197_at	AT1G12900	Glyceraldehyde 3-phosphate dehydrogenase, putative	0.99 P	2.06 P	0.48[e]	0.69 A	0.53 A		0.70[d]	0.26[e]	0.86 A	2.36 P	0.37[e]	0.89 A	1.13 A			0.48[e]
256825_at	AT3G22120	Protease inhibitor/seed storage/LTP family	1.01 P	1.11 P	0.91	0.66 P	0.31 P	1.30	0.65[d]	0.46[e]	1.23 M	1.12 P	1.12	0.83 A	0.10 A	8.17[a]		0.09[e]
262586_at	AT1G15480	DNA-binding protein, putative	1.32 P	1.09 P	1.21	0.61 P	0.14 A	4.42[b]	0.46[e]	0.13[e]	1.59 P	1.35 P	1.18	0.33 P	0.51 A	0.64[d]	0.21[e]	0.38[e]
253060_at	AT4G37710	VQ motif-containing protein	8.73 P	0.90 A	9.68[a]	4.01 A	0.63 A		0.46[e]		9.59 P	0.49 A	19.74[a]	4.75 P	0.38 A	12.57[a]	0.50[e]	
264718_at	AT1G70130	Lectin protein kinase, putative	4.86 P	0.45 A	10.93[a]	1.08 A	0.44 A		0.22[e]		6.94 P	1.23 A	5.64[a]	2.99 A	0.92 A		0.43[e]	

Microarray analysis indicated that, apart from CpNifS, only the SMT homolog HMT-3 and a few other genes were differentially regulated by Se in the CpNifS roots compared to wild type. The higher CpNifS levels may explain the enhanced Se tolerance via prevention of Se incorporation into proteins, as discussed earlier. It is not known whether the HMT-3 protein has SMT activity. Higher SMT activity would be expected to also shuttle Se away from incorporation into proteins, via methylation of SeCys (Neuhierl et al., 1999; Ranocha et al., 2000). The HMT-3 levels were higher under control conditions in CpNifS roots than in wild type, but decreased in response to selenate in the CpNifS plants and not in wild type. Thus, HMT-3 did not appear to contribute to the enhanced Se tolerance in the CpNifS plants. None of the other differentially expressed genes offer an obvious explanation for the higher Se tolerance or accumulation properties of the CpNifS transgenics, but for many of them their functions are not quite known. Thus, from what is known currently it appears that CpNifS affects Se tolerance and accumulation directly rather than via pleiotropic effects on other transcript levels, illustrating the importance of this gene for selenate tolerance. However, as mentioned, it cannot be excluded that there were additional transcriptome differences in the shoot that caused the enhanced tolerance and accumulation in CpNifS plants.

In conclusion, overexpression of CpNifS mitigated Se toxicity and enhanced Se accumulation. The overexpression of CpNifS decreased the amount of nonspecific incorporation of Se in protein, allowing the transgenic plants to tolerate and accumulate more Se

Table II. *The effect of Se on S metabolism*

Genes encoding sulfate transporters and genes for the synthesis of Cys, Met, and glutathione were selected from the transcriptome data of wild-type (Ws) and CpNifS overexpressor (NifS55 and NifS38) plants, described as set 1 and set 2 in Table I. The data of 24 h no-S treatment (−S) and the control S-replete culture at the same time point (+S) appear as references (set A and set B; Maruyama-Nakashita et al., 2005; http://arabidopsis.org/info/expression/ATGenExpress.jsp). Columbia-0 ecotype (Col) was used for the S limitation experiment. The up- and down-regulation by Se or by S limitation were calculated as relative ratios of normalized signals (Se/MS and −S/+S), and displayed using the same superscript letters as in Table I. The relative ratios are calculated for the pairs having "present" call at least in one sample.

Affy Code	AGI Locus	Annotation	Gene Name	Set 1						Set 2						Set A			Set B		
				Ws			NifS55			Ws			NifS38			Col			Col		
				Se	MS	Se/MS	Se	MS	Se/MS	Se	MS	Se/MS	Se	MS	Se/MS	−S	+S	−S/+S	−S	+S	−S/+S
Sulfate transport																					
255105_at	AT4G08620	Sulfate transporter	SULTR1;1	2.69 P	0.29 P	9.33[a]	2.45 P	0.59 P	4.15[a]	2.40 P	0.21 P	11.41[a]	3.33 P	0.25 P	13.59[a]	1.41 P	0.16 P	9.04[a]	1.90 P	0.13 P	15.10[a]
262133_at	AT1G78000	Sulfate transporter	SULTR1;2	1.15 P	0.65 P	1.76[c]	1.08 P	0.98 P	1.10	1.14 P	0.69 P	1.64[c]	1.23 P	0.69 P	1.78[c]	1.15 P	0.73 P	1.58[c]	1.02 P	0.72 P	1.42[c]
255958_at	AT1G22150	Sulfate transporter	SULTR1;3	0.59 A	1.74 A		0.53 A	0.49 A		0.64 A	0.27 A		0.54 A	0.90 A		1.01 A	0.77 A		0.74 A	0.20 A	
250475_at	AT5G10180	Sulfate transporter	SULTR2;1	1.60 P	0.34 P	5.35[a]	1.79 P	0.23 P	7.76[a]	2.03 P	0.27 P	7.59[a]	1.66 P	0.27 P	6.11[a]	2.33 P	0.24 P	9.60[a]	2.75 P	0.31 P	8.94[a]
262134_at	AT1G77990	Sulfate transporter	SULTR2;2	1.16 P	1.14 P	1.02	1.31 P	1.04 P	1.26	0.97 P	0.89 P	1.10	1.03 P	0.72 A	1.42[c]	1.16 P	0.87 P	1.34[c]	0.85 P	0.76 A	1.12
246310_at	AT3G51895	Sulfate transporter	SULTR3;1	1.05 P	1.03 P	1.02	0.74 P	0.66 P	1.11	1.39 P	1.05 P	1.33[c]	0.87 P	0.89 P	0.98	1.42 P	0.97 P	1.47[c]	1.10 P	0.65 P	1.69[c]
253443_at	AT4G02700	Sulfate transporter	SULTR3;2	0.83 A	0.75 A		0.80 A	1.01 A		1.10 A	1.00 A		0.99 A	1.06 A		1.16 A	1.00 A		1.07 A	0.75 M	
264901_at	AT1G23090	Sulfate transporter	SULTR3;3	0.72 P	1.41 P	0.51[d]	1.36 P	1.82 P	0.75[d]	0.78 P	1.32 P	0.59[d]	1.16 P	1.43 P	0.81	0.83 P	0.84 P	0.99	0.83 P	0.84 P	0.98
258267_at	AT3G15990	Sulfate transporter	SULTR3;4	1.45 P	0.91 P	1.59[c]	1.27 P	1.07 P	1.19	1.62 P	1.14 P	1.42[c]	1.45 P	0.93 P	1.56[c]	0.69 P	0.46 P	1.50[c]	0.66 P	0.48 P	1.37[c]
245912_at	AT5G19600	Sulfate transporter	SULTR3;5	0.88 P	0.96 P	0.92	0.93 P	1.02 P	0.91	0.85 P	0.98 P	0.89	0.88 P	1.19 P	0.74[d]	1.13 P	1.36 P	0.83	1.19 P	1.28 P	0.92
245855_at	AT5G13550	Sulfate transporter	SULTR4;1	1.33 P	0.81 P	1.65[c]	1.24 P	0.88 P	1.42[c]	1.24 P	0.73 P	1.71[c]	1.26 P	0.63 P	2.00[c]	1.26 P	0.81 P	1.56[c]	1.12 P	0.60 P	1.88[c]
256244_at	AT3G12520	Sulfate transporter	SULTR4;2	1.90 P	0.58 P	3.30[b]	2.01 P	0.67 P	2.99[b]	1.76 P	0.67 P	2.63[b]	2.05 P	0.56 P	3.63[b]	1.40 P	0.34 P	4.18[a]	1.33 P	0.41 P	3.27[b]
Sulfate reduction																					
256835_at	AT3G22890	ATP sulfurylase	APS1	1.05 P	0.65 P	1.60[c]	0.96 P	0.62 P	1.54	0.99 P	0.60 P	1.62[c]	1.01 P	0.62 P	1.61[c]	1.70 P	1.32 P	1.29	1.97 P	1.79 P	1.10
255785_at	AT1G19920	ATP sulfurylase	APS2	0.62 P	1.12 P	0.56[d]	0.75 P	1.00 P	0.75	0.69 P	1.27 P	0.54[d]	0.72 P	1.17 P	0.61[d]	0.66 P	1.66 P	0.63[d]	1.01 P	1.28 P	0.79
245254_at	AT4G14680	ATP sulfurylase	APS3	1.99 P	0.37 A	5.40[a]	1.27 P	0.32 A	4.00[b]	2.12 P	0.33 A	6.34[a]	1.69 P	0.47 M	3.59[b]	1.19 P	0.62 A	1.94[c]	1.39 P	0.81 P	1.72[c]
249112_at	AT5G43780	ATP sulfurylase	APS4	0.27 P	1.14 P	0.23[e]	0.30 P	1.14 P	0.27[e]	0.26 P	1.03 P	0.25[e]	0.28 P	1.04 P	0.27[e]	0.81 P	1.14 P	0.72[d]	0.97 P	1.46 P	0.67[d]
255284_at	AT4G04610	APS reductase	APR1	1.02 P	0.32 P	3.24[b]	1.08 P	0.38 P	2.87[b]	0.94 P	0.32 P	2.96[b]	1.31 P	0.35 P	3.80[b]	1.02 P	0.99 P	1.03	1.22 P	1.58 P	0.88
264745_at	AT1G62180	APS reductase	APR2	1.11 P	0.50 P	2.23[b]	1.18 P	0.40 P	2.93[b]	1.16 P	0.39 P	2.94[b]	1.28 P	0.43 P	2.97[b]	1.36 P	0.77 P	1.79[c]	1.49 P	0.86 P	1.67[c]
254343_at	AT4G21990	APS reductase	APR3	2.10 P	0.42 P	5.06[a]	1.91 P	0.47 P	4.11[b]	2.41 P	0.48 P	5.07[a]	2.30 P	0.44 P	5.18[a]	1.60 P	0.57 P	2.82[b]	1.36 P	0.62 P	2.21[b]
267112_at	AT2G14750	APS kinase	AKN1	1.11 P	0.89 P	1.26	1.10 P	0.95 P	1.16	0.76 P	0.73 P	1.03	1.05 P	0.68 P	1.55[c]	1.17 P	1.09 P	1.07	0.81 P	1.47 P	0.55[c]
252870_at	AT4G39940	APS kinase	AKN2	1.17 P	0.99 P	1.19	1.10 P	1.01 P	1.09	0.99 P	0.76 P	1.30	1.02 P	0.80 P	1.27	0.83 P	1.63 P	0.51[d]	0.67 P	2.25 P	0.30[e]
259339_at	AT3G03900	APS kinase	AKN3	0.74 P	1.08 P	0.69[d]	0.94 P	0.98 P	0.96	1.14 P	0.95 P	1.20	1.11 P	0.98 P	1.13	1.18 P	1.22 P	0.97	0.93 P	1.02 P	0.93
247005_at	AT5G67520	APS kinase	AKN4	0.98 P	0.93 P	1.05	1.02 P	1.15 P	0.89	0.96 P	0.98 P	1.00	0.69 P	0.68 P	1.01	1.20 P	1.63 P	0.74[d]	1.31 P	1.08 P	1.21
250846_at	AT3G04390	Sulfite reductase	SIR	1.03 P	0.90 P	1.15	1.21 P	0.82 P	1.47[c]	1.21 P	0.90 P	1.35[c]	1.09 P	0.85 P	1.29	0.93 P	1.05 P	0.88	0.97 P	1.21 P	0.80
Cys synthesis																					
245286_at	AT4G14880	O-acetylserine (thiol) lyase	Bsas1;1	1.00 P	1.11 P	0.90	1.00 P	1.35 P	0.74[d]	1.03 P	1.10 P	0.93	0.98 P	1.11 P	0.88	0.77 P	0.90 P	0.85	0.72 P	0.79 P	0.92
256930_at	AT3G22460	O-acetylserine (thiol) lyase	Bsas1;4	0.95 P	1.54 P	0.62[d]	1.23 P	2.17 P	0.57[d]	0.69 P	1.45 P	0.48[d]	1.05 P	1.17 P	0.90	0.81 P	0.81 P	1.00	0.94 P	0.92 P	1.03
260566_at	AT2G43750	O-acetylserine (thiol) lyase	Bsas2;1	1.38 P	1.00 P	1.36[c]	1.12 P	0.90 P	1.25	1.49 P	0.95 P	1.57[c]	1.21 P	1.00 P	1.21	1.01 P	0.87 P	1.16	0.87 P	0.91 P	0.96
251467_at	AT3G59760	O-acetylserine (thiol) lyase	Bsas2;2	1.01 P	0.97 P	1.04	0.96 P	1.00 P	0.96	0.94 P	1.09 P	0.86	1.06 P	1.00 P	1.06	0.85 P	1.11 P	0.77	0.84 P	1.01 P	0.83
251322_at	AT3G61440	O-acetylserine (thiol) lyase	Bsas3;1	1.13 P	1.18 P	0.95	1.02 P	1.24 P	0.82	0.96 P	1.11 P	0.88	0.98 P	1.04 P	0.94	0.94 P	0.94 P	1.00	0.72 P	0.98 P	0.82
259094_at	AT3G04940	O-acetylserine (thiol) lyase	Bsas4;1	1.03 P	1.17 P	0.88	0.84 P	0.97 P	0.87	0.86 P	1.07 P	0.81	0.76 P	0.90 P	0.84	1.15 P	0.90 P	1.27	1.03 P	1.31 P	0.79
246701_at	AT5G28020	O-acetylserine (thiol) lyase	Bsas4;2	1.75 P	0.98 P	1.78[c]	1.58 P	1.15 P	1.37[c]	1.66 P	1.02 P	1.62[c]	1.51 P	0.87 P	1.74[c]	0.81 P	0.83 P	0.98	0.86 P	0.65 P	1.34[c]
246700_at	AT5G28030	O-acetylserine (thiol) lyase	Bsas4;3	0.24 A	0.14 A		1.77 A	1.01 A		1.60 A	0.38 A		1.06 A	0.88 A		1.24 A	1.06 A		0.99 A	0.19 A	
259172_at	AT3G03630	O-acetylserine (thiol) lyase	Bsas5;1	1.04 A	0.71 P	1.47[c]	0.93 A	1.00 A	0.93	1.00 A	1.14 P	0.88	1.05 A	0.85 A	1.23	0.91 A	0.93 A	0.98	1.57 A	1.09 P	1.44[c]
247982_at	AT5G56760	Ser acetyl-transferase	Sera1;1	0.92 P	1.04 P	0.89	0.91 P	1.00 P	0.91	0.87 P	1.00 P	0.87	0.94 P	0.91 P	1.04	1.99 P	1.83 P	1.09	2.00 P	2.04 P	0.98
260602_at	AT1G55920	Ser acetyl-transferase	Sera2;1	1.46 P	1.22 P	1.20	1.28 P	1.52 P	0.84	1.11 P	0.94 P	1.18	1.06 P	0.86 P	1.23	0.82 P	0.63 P	1.30	0.48 P	0.48 P	1.01
257194_at	AT3G13110	Ser acetyl-transferase	Sera2;2	1.23 P	1.17 P	1.05	0.93 P	1.23 P	0.76	0.78 P	0.84 P	0.93	0.75 P	0.90 P	0.83	0.98 P	1.07 P	0.91	1.02 P	1.13 P	0.90
264594_at	AT2G17640	Ser acetyl-transferase	Sera3;1	2.24 P	0.96 P	2.29[b]	2.22 P	0.95 P	2.34[b]	1.86 P	0.97 P	1.93[c]	1.93 P	0.72 P	2.67[b]	1.47 P	0.79 P	1.87[c]	1.02 P	0.51 P	2.03[b]

(*Table continues on following page.*)

Table II. *(Continued from previous page.)*

Affy Code	AGI Locus	Annotation	Gene Name	Set 1						Set 2						Set A			Set B		
				Ws			Nifs35			Ws			Nifs38			Col			Col		
				Se	MS	Se/MS	Se	MS	Se/MS	Se	MS	Se/MS	Se	MS	Se/MS	−S	+S	−S/+S	−S	+S	−S/+S
GSH and Met synthesis																					
254270_at	AT4G23100	γ-Glutamyl-cysteine synthetase	GSH1	1.02 P	1.52 P	0.67[d]	1.07 P	1.67 P	0.64[d]	0.92 P	1.45 P	0.64[d]	0.92 P	1.38 P	0.66[c]	0.75 P	0.89 P	0.84	0.69 P	0.98 P	0.71[d]
246785_at	AT5G27380	Glutathione synthetase	GSH2	1.22 P	1.16 P	1.05	1.12 P	1.03 P	1.09	1.11 P	0.97 P	1.14	1.07 P	0.90 P	1.19	0.70 P	0.63 P	1.10	0.66 P	0.82 P	0.80
259279_at	AT3G01120	Cystathionine γ-synthase	CGS	0.97 P	1.12 P	0.87	0.85 P	1.07 P	0.80	0.90 P	1.03 P	0.87	0.78 P	1.12 P	0.70[d]	0.83 P	1.04 P	0.80	0.73 P	1.05 P	0.70[d]
256531_at	AT1G33320	Cystathionine γ-synthase, putative		0.79 A	1.52 A		0.96 A	1.40 A		0.78 A	0.72 A		1.04 A	0.90 A		1.25 A	1.43 A		0.90 A	1.29 A	
251666_at	AT3G57050	Cystathionine β-lyase	CBL	1.19 P	1.19 P	0.99	1.01 P	1.03 P	0.98	0.87 P	0.99 P	0.88	0.91 P	0.94 P	0.96	0.83 P	1.06 P	0.78	0.95 P	1.32 P	0.72[d]
246185_at	AT5G20980	Met synthase		0.98 P	0.86 P	1.14	0.61 P	0.78 P	1.05	1.02 P	0.88 P	1.17	0.95 P	1.17 P	0.81	1.44 P	1.54 P	0.94	1.41 P	1.22 P	1.16
259343_s_at	AT3G03780	Met synthase		0.95 P	0.93 P	1.02	0.86 P	0.94 P	0.94	0.99 P	1.01 P	0.97	0.99 P	1.01 P	0.98	1.80 P	1.74 P	1.04	1.45 P	1.61 P	0.90
Met metabolism																					
261937_at	AT1G64660	Cystathionine γ-lyase, putative		1.63 P	1.06 P	1.54[c]	1.69 P	1.09 P	1.55[c]	1.77 P	0.94 P	1.88[c]	1.56 P	0.94 P	1.66[c]	0.43 P	0.26 P	1.76[c]	0.38 P	0.21 P	1.85[c]
260594_at	AT1G55880	Cystathionine β-synthase, putative		0.98 P	1.32 P	0.75[d]	1.00 P	1.11 P	0.90	0.89 P	1.09 P	0.81	0.80 P	0.94 A	0.85	1.00 P	0.80 A	1.26	1.02 A	1.04 P	0.98
260913_at	AT1G02500	S-adenosyl-methionine synthetase	SAM1	1.06 P	1.16 P	0.92	0.94 P	1.31 P	0.71[d]	1.15 P	1.09 P	1.05	0.93 P	1.19 P	0.78	0.94P	0.86 P	1.06	0.75 P	0.88 P	0.85
255552_at	AT4G01850	S-adenosyl-methionine synthetase	SAM2	1.23 P	1.38 P	0.89	0.99 P	1.25 P	0.79	0.87 P	1.10 P	0.88	0.90 P	1.15 P	0.78	1.01P	0.77 P	1.32	0.76 P	0.86 P	0.89
263836_at	AT2G36880	S-adenosyl-methionine synthetase	SAM3	1.12 P	1.24 P	0.90	0.97 P	1.19 P	0.82	0.93 P	1.06 P	0.88	0.86 P	0.99 P	0.87	1.09P	0.87 P	1.26	0.85 P	1.01 P	0.84
258415_at	AT3G17390	S-adenosyl-methionine synthetase		1.46 P	1.05 P	1.39[c]	1.16 P	0.89 P	1.31	1.34 P	0.94 P	1.42[c]	1.06 P	0.89 P	1.19	1.26P	0.79 P	1.59[c]	0.95 P	0.87 P	1.10
245356_at	AT4G13940	S-adenosyl-homocysteine hydrolase		1.22 P	1.22 P	1.00	0.94 P	1.16 P	0.81	1.18 P	1.05 P	1.12	0.91 P	1.12 P	0.82	0.95P	0.78 P	1.21	0.71 P	0.84 P	0.84
257173_at	AT3G23810	S-adenosyl-homocysteine hydrolase		0.86 P	1.00 P	0.86	0.82 P	0.92 P	0.88	0.93 P	1.00 P	0.93	0.82 P	1.13 P	0.73[d]	1.80P	1.55 P	1.16	1.60 P	1.71 P	0.94
258075_at	AT3G25900	Homocysteine S-methyl-transferase	HMT-1	1.13 P	0.89 P	1.28	1.07 P	1.02 P	1.05	0.98 P	0.83 P	1.18	0.89 P	0.83 P	1.08	1.07P	0.86 P	1.25	1.20 P	1.21 P	0.99
251175_at	AT3G63250	Homocysteine S-methyl-transferase	HMT-2	1.17 P	1.15 P	1.02	1.05 P	0.95 P	1.11	1.11 P	1.17 P	0.95	0.91 P	1.08 P	0.84	0.71P	0.76 P	0.93	0.86 P	0.84 P	1.03
258322_at	AT3G22740	Homocysteine S-methyl-transferase	HMT-3	1.00 P	1.10 P	0.90	0.64 P	1.58 P	0.41[e]	1.01 A	0.97 P	1.03	0.73 A	1.33 P	0.55[d]	0.69A	2.02 P	0.44[e]	0.93 A	2.22 P	0.42[e]
Se-binding protein																					
245285_s_at	AT4G14030	Se-binding protein, putative		1.102 P	0.446 P	2.47[b]	1.165 P	0.635 P	1.83[c]	0.986 P	0.573 P	1.72[c]	1.28 P	0.49 P	2.61[b]	1.314P	1.081 P	1.22	1.014 P	0.862 P	1.15
245285_s_at	AT4G14040	Se-binding protein, putative																			
257197_at	AT3G23800	Se-binding protein, putative		0.59 P	1.369 P	0.43[e]	0.743 P	1.386 P	0.54[d]	0.505 P	1.22 P	0.41[e]	0.64 P	1.139 P	0.56[d]	0.861P	1.215 P	0.71[d]	0.839 P	1.629 P	0.52[d]

compared to wild-type Arabidopsis. Overexpression of CpNifS also affected S metabolism, enhancing S uptake. Microarray analysis helped explain some of the observed physiological effects of selenate. The paucity of transcripts that were differentially regulated in the transgenics compared to wild type on selenate suggests that overexpression of CpNifS is likely the direct reason why the transgenics are more tolerant to and accumulate more Se. CpNifS-overexpressing transgenic plants that have enhanced ability to tolerate and accumulate Se may ultimately be useful in phytoremediation—the use of plants to clean polluted soils. For that purpose, the same gene could be overexpressed in a high biomass species, such as Indian mustard. Plants with enhanced Se accumulation may also have value as fortified foods, since Se-enriched diets can help prevent cancer and other detrimental effects of oxidative stress.

MATERIALS AND METHODS

Overexpression of CpNifS

The CpNifS (At1g18490) coding sequence (Pilon-Smits et al., 2002) was cloned as a *Nco*I/*Bam*HI fragment into pMOG18 (Sijmons et al., 1990) under control of the constitutive 35S cauliflower mosaic virus promoter, before transfer into the plant binary vector pBART containing the glufosinate-ammonium resistance marker. Sequence analysis confirmed the final construct in which the *Hin*dIII/*Eco*RI sites were replaced by *Not*I sites. The construct was then transferred into *Agrobacterium tumefaciens* (strain C58C1) and transformed into Arabidopsis (*Arabidopsis thaliana*; ecotype Wassilewskija [Ws]) using the floral dip method (Clough and Bent, 1998). Glufosinate-resistant lines were selected on half-strength MS (Sigma-Aldrich; Murashige and Skoog, 1962) agar media with 1% (w/v) Suc and 5 mg L^{-1} glufosinate-ammonium and transferred to soil. Plants were grown in Metromix potting soil under controlled conditions (light intensity of 40 μmol m^{-2} s^{-1}, 16-h-light/8-h-dark cycle at 24°C). After 2 weeks, PCR analysis was used to confirm insertion of the gene construct by using 5′-ccttcgcaagacccttcctc and 5′-ggaagacagtttctcgtacaaat primers that hybridize to the 35S CaMV promoter and CpNifS sequence, respectively (Fig. 1). Six lines showed the expected PCR product, while no PCR product was detected in the wild-type control (data not shown). The six transgenic lines were propagated to homozygosity and did not show any phenotypic differences compared to wild type on control media and on soil.

Immunoblot analysis was used to determine the degree of overexpression of CpNifS in the transgenics. Shoot and root proteins from plants grown on half-strength MS media were separated by SDS-PAGE and transferred to nitrocellulose by electroblotting. The CpNifS protein was immunodetected using polyclonal antibodies raised in chicken against CpNifS protein (Pilon-Smits et al., 2002). Two transgenic lines, CpNifS38 and CpNifS55, were selected for further analysis. Protein concentration of wild type and the transgenics were determined using a desktop scanner and ImageJ imaging software (National Institute of Health; http://rsb.info.nih.gov/ij/).

Chloroplast isolation was performed as described by Rensink et al. (1998) from the two selected lines and wild type grown on soil for 3 weeks. A western blot was used to determine if CpNifS was localized to the stromal fraction of chloroplasts; 3 μg of chlorophyll were loaded per lane. Antibodies raised against plastocyanin were also used on blots from the same gel as controls for uniform loading.

Se and S Metabolism

Se tolerance was determined by measuring the root length of seedlings (n = 30) grown for 10 d on vertical plates containing half-strength MS agar media supplemented with 1% (w/v) Suc with or without selenate (40 μM Na_2SeO_4) as described by Pilon-Smits et al. (1999). Total Se accumulation was assayed by growing seedlings (n = 100) for 20 d on horizontal plates containing half-strength MS agar containing Suc media with or without 40 μM Na_2SeO_4. Roots and shoots were harvested, separated, washed to remove any external Se, and dried overnight at 70°C. Samples from pooled root samples (n = 3) and shoot samples (n = 5) were then acid digested and analyzed by inductively coupled plasma-atomic emission spectrometry as described (Pilon-Smits et al., 1999). Incorporation of Se in protein was determined in seedlings (n = 300) grown for 14 d on agar medium supplied with 20 μM SeO_4 as described by Pilon et al. (2003).

GeneChip Hybridization and Microarray Analysis

Plants were grown in a growth chamber for 10 d on vertical plates with or without 40 μM Na_2SeO_4. For each line, total RNA was extracted from the roots using the RNeasy Plant Mini kit (Qiagen). The technical manual of the Arabidopsis Genome GeneChip array (Affymetrix) was used in preparing the labeled target cRNA. Double-stranded cDNA was prepared from 20 μg of total RNA using the Superscript Choice system (Invitrogen) and transcribed in vitro using the BioArray High Yield RNA transcript kit (Enzo). After purification and fragmentation, the labeled cRNA was hybridized to an Arabidopsis Genome Chip Array containing more than 22,500 probe sets representing approximately 24,000 genes and placed in a Hybridization Oven model 640 (Affymetrix). Washing and staining of the chips were carried out using GeneChip Fluidics Station model 400. Scanning was performed with the Gene Array Scanner (Agilent Technologies), and the Microarray Suite 5.0 (Affymetrix) and Gene Spring 7.2 (Silicon Genetics) were used for data analysis as described (Maruyama-Nakashita et al., 2003; 2005). Raw signals of each transcript were normalized with the median of all measurements on the chip, from which fold changes of signal intensities were calculated.

μXRF/μXAS

Whole-plant samples grown for 3 weeks on half-strength MS medium with 40 μM selenate were washed to remove any external Se, and shipped on dry ice to the Advanced Light Source at the Lawrence Berkeley Laboratory for analysis on Beamline 10.3.2 (Marcus et al., 2004). Intact mature leaves were mounted using silicone grease to a Peltier stage and kept at −30°C to reduce radiation damage. Crude mapping of the Se concentration was performed on one representative leaf for each plant type, with a 16 × 7 μm beam sampled in 12 × 12 μm pixels, followed by fine mapping (5 × 5 μm beam, 3 × 3 μm pixels) on at least five selected areas per leaf, all at 12,859 eV. Se K-edge XANES was measured to determine the Se oxidation state at specific points in the fine map (Pickering et al., 1999). A selenate solution was used as a calibration standard.

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Foreword to Chapter 4

In the last chapter, I showed that the manipulation of one gene (CpNifS) could augment selenium tolerance and accumulation. A microarray analysis between WT and transgenic CpNifS plants showed little difference in how these plants' transcriptome respond to selenate stress. Nonetheless, I became excited about the possibility of identifying other genes that could also lead to increased selenium tolerance and accumulation in plants. Chapter 4 was conceived when I questioned if other genes besides CpNifS are involved in conferring tolerance to selenate. To help answer this question, I again used microarray analysis to help determine if certain transcripts are differentially regulated in WT Arabidopsis plants when treated with selenate.

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Transcriptome analyses give insights into selenium-stress responses and selenium tolerance mechanisms in Arabidopsis

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Selenate is chemically similar to sulfate and can be taken up and assimilated by plants via the same transporters and enzymes. In contrast to many other organisms, selenium (Se) has not been shown to be essential for higher plants. In excess, Se is toxic and restricts development. Both Se deficiency and toxicity pose problems worldwide. To obtain better insights into the effects of Se on plant metabolism and into plant mechanisms involved in Se tolerance, the transcriptome of Arabidopsis plants grown with or without selenate was studied and Se-responsive genes identified. Roots and shoots exhibited different Se-related changes in gene regulation and metabolism. Many genes involved in sulfur (S) uptake and assimilation were upregulated. Accordingly, Se treatment enhanced sulfate levels in plants, but the quantity of organic S metabolites decreased. Transcripts regulating the synthesis and signaling of ethylene and jasmonic acid were also upregulated by Se. Arabidopsis mutants defective in ethylene or jasmonate response pathways exhibited reduced tolerance to Se, suggesting an important role for these two stress hormones in Se tolerance. Selenate upregulated a variety of transcripts that were also reportedly induced by salt and osmotic stress. Selenate appeared to repress plant development, as suggested by the downregulation of genes involved in cell wall synthesis and auxin-regulated proteins. The Se-responsive genes discovered in this study may help create plants that can better tolerate and accumulate Se, which may enhance the effectiveness of Se phytoremediation or serve as Se-fortified food.

Introduction

Selenium (Se) is a naturally occurring element commonly found in Cretaceous shale rock. When extensively weathered or irrigated, these sediments have the potential to leach Se into groundwater where it can become a source of toxicity for livestock (Fessler et al. 2003), wildlife (Hamilton 2004) and humans (Hira et al. 2004). At the same time, Se is an essential micronutrient for many animals and bacteria. To date, there is no evidence that vascular plants need Se for survival, although it may promote plant growth in some cases (Beath et al. 1934, Galeas et al. 2007).

Selenium is chemically similar to sulfur (S) and can be metabolized by S metabolic pathways. Plants take up

Abbreviations – ACC, 1-aminocyclopropane 1-carboxylate; APS, ATP sulfurylase; AsA, ascorbic acid; CBF, cold-binding factor; DHA, dehydroascorbic acid; ERF, ethylene responsive factor; HSF, heat shock factor; HSP, heat shock protein; JA, jasmonic acid; LEA, late embryogenic abundant; Pdf, plant defensin; qRT-PCR, quantitative real-time polymerase chain reaction; WT, wild-type.

selenate, the most common soluble form of Se in soil, inadvertently via sulfate transporters and, presumably also inadvertently, assimilate it into selenocysteine and selenomethionine (Terry et al. 2000). Non-specific replacement of the two essential S amino acids, cysteine and methionine, by these Se analogues in proteins is toxic (Stadtman 1990, Terry et al. 2000). Selenium-accumulating plants may have potential as fortified food with enhanced nutritional quality or for cleanup of soils in which Se has accumulated to dangerous levels. To enhance plants' capacity for Se accumulation, several genes involved in S assimilation have been targets of genetic manipulation (for a review, see Pilon-Smits 2005). Recently, *Brassica juncea* (Indian mustard) plants overexpressing ATP sulfurylase (APS) or selenocysteine lyase have shown enhanced capacity to accumulate Se from polluted soil in field studies (Banuelos et al. 2005, 2007). Thus, although Se is a concern for public health and agriculture, plants can have the capacity to attenuate the toxic effects of Se.

It is likely that in addition to S assimilation, other pathways are involved in a plant's natural capacity to accumulate and tolerate Se. If these pathways could be identified, it may accelerate the success of using transgenic plants to remediate Se-contaminated soils. Recently, overexpression of a putative Se-binding protein enhanced selenate tolerance in Arabidopsis (Agalou et al. 2005). At this point, relatively little is known about plants' defensive response to Se, and even less is known about how selenate modulates gene expression. In the future, biologists will likely increasingly use a systems approach to understand how the complexity in transcriptomics, proteomics and metabolomics relates to the physiology of the plant growing under stress (Hesse and Hoefgen 2006). To help meet this challenge, the transcriptome of Arabidopsis grown with or without selenate was studied in order to identify Se-responsive genes. The effects of Se on the transcriptome were compared with the responses reported earlier of plants grown under other abiotic stresses, e.g. salt and osmotic stress. Because the microarray analysis suggested that Se induced S deficiency, the effect of Se on several S metabolites was further investigated. Also, because the microarray studies suggested that ethylene and jasmonic acid (JA) might play a role in plant responses to Se, several mutants with reduced or elevated levels of these hormones were investigated for Se tolerance. Together, these studies provide a better understanding of the mechanisms *Arabidopsis thaliana* uses to deal with selenate stress and may give an insight into how to further increase Se tolerance and accumulation in plants suitable for phytoremediation.

Materials and methods

Unless otherwise described, Arabidopsis plants (ecotype wassilewskija) were grown in a growth chamber (150 μmol m^{-2} s^{-1} PAR, 16 h light/8 h dark cycle, 24°C) on vertically placed agar plates containing 0.5 MS medium and 1% sucrose (Murashige and Skoog 1962). Plants were grown on growth plates with or without 40 μ*M* selenate. Selenate constitutes the majority of bioavailable Se in soils, and the concentration of selenate in the growth plates (3.2 ppm) is naturally found in seleniferous soils (Galeas et al. 2007). Plants were grown for 10 days, at which point there was a clear difference in root length grown with or without selenate, while plant growth was not limited by plate size yet.

For the microarray studies, roots and shoots were separated and analyzed independently. Total RNA was extracted using the RNeasy Plant Mini kit (Qiagen, Valencia, CA). Synthesis of cDNA, hybridization to an Arabidopsis Genome Chip (Affymetrix) containing probes for 24 000 genes and scanning of the Genome Chip have been described (Van Hoewyk et al. 2005). Two biological replicates for each treatment were performed, each consisting of 20–25 pooled plants. Preprocessing of the raw data and calculation of transcript signals were performed using Affymetrix Microarray Suite 5.0. Signal intensity values were then normalized with the median of all measurements. All further analysis was conducted in R using BioConductor (www.bioconductor.org). The microarray analysis was performed within the minimal information about microarray experiments (MIAME) guidelines (Brazma et al. 2001). All data sets have been submitted to the Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov).

Roots and shoots were analyzed separately. For each transcript, the fold change was determined and a *P* value was calculated using a moderated *t*-test in limma (Smyth 2004) assuming equal variance between the two groups. Transcripts that were called 'Present' for at least two out of four arrays were retained for further classification. We note that a multiple testing adjustment was not performed because no genes would have been identified as differentially expressed in the shoots. Biological functions of identified Se-responsive genes were categorized (www.arabidopsis.org). Our data set of identified Se-responsive transcripts was compared with previously performed microarray analyses investigating the effect of salt and mannitol (osmotic stress) treatment for 24 h in root and shoot tissue (Kilian et al. 2007).

Macroarrays were performed in duplicate to validate the microarray results. Based on the microarray data, a total of 31 genes representing a range of up- and

downregulation in root and shoot tissues were selected for macroarray comparison. Briefly, root and shoot Arabidopsis RNA was extracted and cDNA was synthesized as described above from plants grown with or without selenate and were hybridized to a nylon membrane containing the selected cDNAs (Tamaoki et al. 2003). A regression analysis was performed to determine how closely the macroarray data matched the microarray data.

Levels of several metabolites in shoot tissue were determined in plants grown as described above with or without selenate for 10 days. Measurement of non-protein thiol levels was performed (in three replicates) using Ellman's reagent as described (Zhu et al. 1999). Amino acid and ammonium analysis were performed by AccQ Tag (Waters, Milford, MA) labeling methods as described in the manufacturer's protocol. Incorporation of S in protein was determined as previously reported (Pilon et al. 2003). Measurements of sulfate and nitrate were performed by ion chromatography using an HS11-HC column according to the manufacturer's specifications (Dionex Coorporatation, Sunnyvale, CA). Reduced and oxidized levels of ascorbic acid (AsA) and glutathione were measured as previously described (Yoshida et al. 2006).

The following transgenic or mutant Arabidopsis lines (ecotype Columbia) were obtained from the Arabidopsis Biological Resource Center (www.arabidopsis.org): ein2-1, accession no. CS3071; ein3-1, accession no. CS8057; jar1-1, accession no. CS8077; an overexpressor of ERF1, accession no. CS6142; and a T-DNA knockout of ACS6, accession no. SALK_090423. The mutant or transgenic lines were compared with wild-type (WT) Columbia for selenate tolerance by measuring the root length of seedlings ($n = 35$) grown for 10 days on vertical plates with or without 40 μM selenate as described (Van Hoewyk et al. 2005). All statistical analyses (except analysis of the transcriptome data) included student's *t*-tests and were performed using the JMP-IN software package (SAS Institute, Cory, NC).

Results and discussion

Identification and functional categorization of Se-responsive transcripts

To better understand how Se changes the Arabidopsis transcriptome, seedlings were grown with or without selenate for 10 days, at which point differences in growth was visually observed (*Supplementary material* Fig. S1 shows the phenotype of the plants at the end of both treatments). This time point was optimal in that it clearly showed the phenotypic differences between Se and control treatments and growth was not limited by plate size. Affymetrix ATH-1 GeneChip arrays containing probes for the Arabidopsis genome were used to identify transcriptome differences in root and shoot tissue in response to selenate. Using a *P* value of less than 0.05 and a minimal fold change of greater than 2, 893 genes were found to be responsive to Se in root tissue; of this set, 340 and 553 genes were down- and upregulated, respectively. In shoot tissue, 385 genes were identified as Se responsive, of which 165 and 220 were down- and upregulated, respectively. The discrepancy in the number of Se-responsive genes in roots and shoots became more accentuated as the fold-change threshold was increased (Table 1). Of the Se-responsive genes identified in roots and shoots, 60 transcripts were differentially regulated in both organs. The complete list of identified genes in root and shoot tissue can be found in *Supplementary material* (Table S1). Possibly, the observation that more Se-responsive genes were identified in roots compared with shoots simply reflects the stress imposed by selenate as it is transported in root cells. The root is in closest contact with the toxic Se and the location where selenate toxicity is first perceived by the plant.

Se-responsive genes whose biological function could be identified were categorized (Fig. 1). Of particular importance is that nearly 50% of both root and shoot upregulated transcripts were categorized as responsive to biotic/abiotic stimuli and stress, providing evidence that selenate is toxic and induces abiotic stress. When comparing regulation between roots and shoots, notable differences include a higher percentage of transcripts involved in transcription in the roots, while the percentage of genes involved in transport was higher in shoots. Among downregulated shoot transcripts, the percentage of genes involved in development (15%) was three-fold greater than any other group, suggesting resources for

Table 1. Genes differentially regulated by selenate were selected based on (A) *P* value and (B) fold change. Arabidopsis plants were grown for 10 days with or without selenate. Root tissue and shoot tissue were harvested and analyzed separately for two independently replicated experiments.

	Roots up	Roots down	Shoots up	Shoots down
Sorted by significance (*P* values)				
P value <0.05, fold change >2	553	340	220	165
P value <0.01, fold change >2	500	286	60	45
P value <0.001, fold change >2	250	63	6	1
Sorted by fold change				
P value <0.05, fold change >2	553	340	220	165
P value <0.05, fold change >3	355	96	80	50
P value <0.05, fold change >4	220	30	30	20

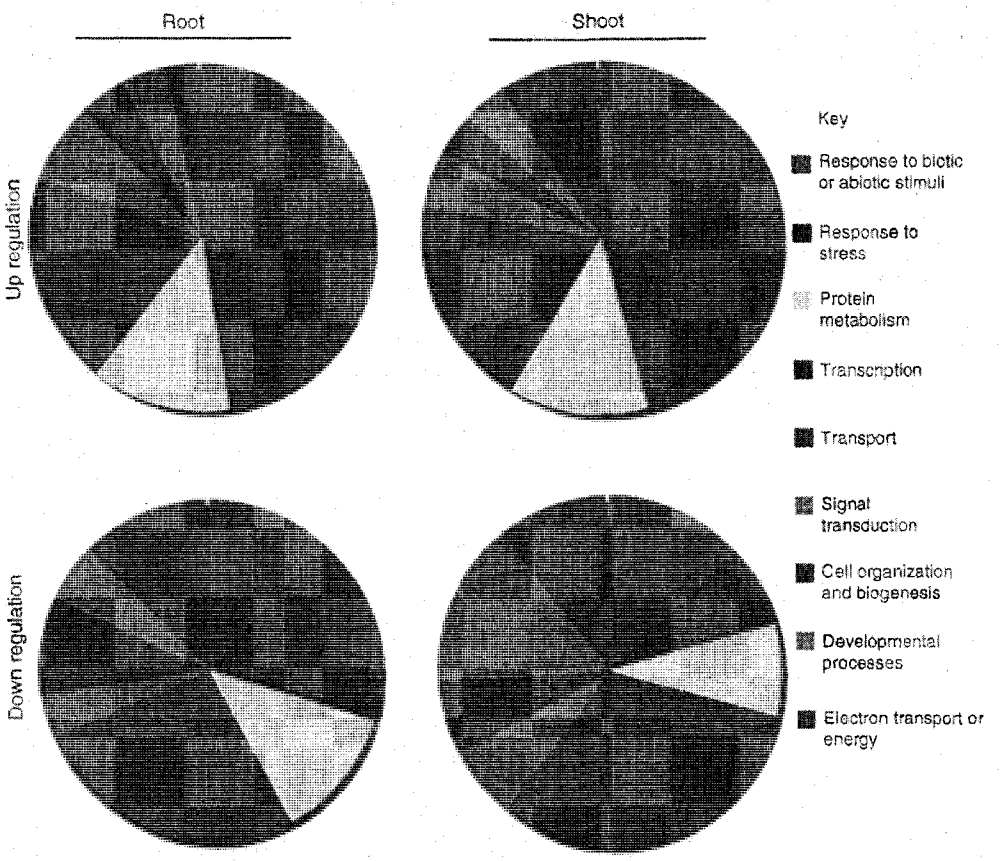

Fig. 1. Identified up- and downregulated selenate-responsive genes ($P < 0.05$, fold change >2 or <0.5) for which the biological function is known (functional groups as proposed by www.arabidopsis.org) and is categorized for root tissue (893 genes) and shoot tissue (385 genes).

growth and development may be reallocated in response to selenate stress. Lastly, the percentage of genes (13%) involved in protein metabolism was roughly consistent in all four groups of both down- and upregulated genes in root and shoots, with the exception of downregulated transcripts in shoots (8%).

Identification of the 50 most significantly Se-responsive genes in root and shoot tissue

Root tissue

The 50 most significantly selenate-responsive genes (according to P value) in root tissue are shown in Table 2 (panel A). Nearly all genes in this list were upregulated, except for a glycine-rich mitochondrial protein (At1g67870) and a plastid carbonic anhydrase (At3g01500). Quite noteworthy from this list are the transcripts involved in calcium signaling, such as a calcium transporter (At5g26220), calmodulin-related proteins (At1g76640, At1g76650 and At2g26530) and a calcium-binding protein (At4g27280). Abiotic stress, including drought, salinity and cold temperature, can increase cytosolic calcium levels and elicit stomatal closure (Allen et al. 2000). Recent evidence suggests that H_2O_2 increases calcium influx in Arabidopsis root cells, particularly in the zone of elongation (Demidchik et al. 2007). It appears that genes involved in calcium signaling are similarly upregulated in response to selenate and activate a defense response.

Transcripts encoding a lipoxygenase (At1g72520) and 1-aminocyclopropane 1-carboxylate (ACC) synthase (ACS6-At4g11280), responsible for the synthesis of JA and ethylene, respectively, were among those upregulated in roots. These two plant hormones are involved in stress response and can upregulate a defensive network by regulating the expression of transcriptional factors. Indeed, seven transcription factors belonging to the ethylene-responsive factor (ERF) family were identified (At244840, At1g74930, At1g19210, At4g34410,

Table 2. The fifty most significantly selenate-responsive transcripts in root tissue and shoot tissue.

Locus	*P* value	Fold change	Annotation
A. Root tissue			
At5g26220	4.02E-06	95.27	Putative ChaA Ca^{2+}/H^{+} cation transport protein
At1g80840	4.88E-06	59.17	WRKY40 pathogen transcription factor, responsive to SA
At1g76640	5.19E-06	359.51	Calmodulin-related protein
At1g76650	5.85E-06	74.57	Calmodulin-related protein
At4g27654	6.93E-06	64.08	Putative protein
At3g60140	9.12E-06	30.76	Induced by senescence, glucosinolates?
At4g29780	9.33E-06	34.60	Putative protein
At2g46400	1.15E-05	25.76	WRKY46 TF
At2g44840	1.23E-05	86.05	ERF B-3, pathogen TF
At1g67870	1.29E-05	0.04	Glycine-rich mitochondrial protein
At1g27730	1.31E-05	29.25	Salt-tolerance zinc-finger protein (ZAT10). Responsive to pathogen and drought
At1g74930	1.42E-05	48.16	DREB subfamily A-5 of ERF/AP2 transcription factor family
At1g72520	1.44E-05	84.25	Lipoxygenase, responsive to JA and wounding
At1g19210	1.59E-05	34.55	DREB subfamily A-5 of ERF/AP2 transcription factor family
At4g34410	1.61E-05	263.44	ERF B-3 of ERF/AP2 transcription factor
At3g44260	1.80E-05	35.14	Cinnamoyl CoA reductase 4-NOT transcription complex protein
At1g12030	1.88E-05	92.94	Putative protein
At5g48850	2.21E-05	33.55	Putative protein
At3g10930	2.29E-05	16.22	Putative protein
At1g15010	2.33E-05	16.23	Putative protein
At4g27652	2.89E-05	15.52	Putative protein
At4g24570	3.01E-05	28.94	Putative mitochondrial uncoupling protein
At2g35930	3.14E-05	10.71	Putative protein
At5g17350	3.16E-05	17.93	Putative protein
At1g74450	3.17E-05	11.79	Putative protein
At2g26530	3.20E-05	14.23	Similar to calmodulin-binding protein
At5g44120	3.31E-05	14.51	12S seed storage protein
At1g61340	3.32E-05	10.93	LEA protein
At5g51190	3.33E-05	14.97	ERF B-3 of ERF/AP2 transcription factor
At4g11280	3.36E-05	16.54	ACC synthase; ethylene synthesis, responsive to JA
At5g38700	3.74E-05	9.59	Putative protein
At1g17750	3.94E-05	9.65	Protein serine/threonine kinase activity
At4g27280	3.95E-05	10.96	Ca++-binding protein
At5g45340	4.01E-05	17.11	Cyt P450, responsive to drought, ABA degradation
At3g01500	4.03E-05	0.03	Chloroplast carbonic anhydrase; carbon utilization
At3g45960	4.41E-05	11.14	Expansin family protein; cell wall
At5g62520	4.86E-05	14.36	Similar to RCD1, responsive to salt, mediates JA and ethylene signaling
At5g12030	4.93E-05	21.56	HSP17.6A, responsive to H_2O_2. Overexpression increases drought tolerance
At2g41640	4.98E-05	9.28	Putative protein
At5g20230	5.00E-05	13.73	Cu-binding protein. Responsive to Al and oxidative stress
At1g28370	5.00E-05	14.74	ERF subfamily B-1 of ERF/AP2 transcription factor
At2g25735	5.74E-05	22.60	Putative protein
At1g22810	5.96E-05	9.06	DREB subfamily A-5 of ERF/AP2 transcription factor
At1g28370	7.76E-05	14.74	ERF subfamily B-1 of ERF/AP2 transcription factor
At5g57560	6.04E-05	10.53	Endoxyloglucan transferase (TCH4); cell wall genesis
At4g14680	6.04E-05	5.91	ATP sulfurylase; sulfur assimilation
At3g50060	6.37E-05	8.57	MYB transcription factor, responsive to Cd, salt, SA, JA, ethylene
At5g59820	6.38E-05	7.55	Zinc-finger protein, responsive to cold, salt, oxidative stress
At1g59860	7.05E-05	10.10	HSP17.6A–C
At1g23730	7.30E-05	45.08	Putative carbonic anhydrase
B. Shoot tissue			
At1g56600	5.43E-04	18.80	Galactinol synthase, responsive to drought
At2g36750	6.84E-04	7.57	Glycosyltransferase responsive to H_2O_2
At2g37770	7.47E-04	5.13	Aldo/keto reductase family protein

Table 2. Continued.

AtMg00040	7.73E-04	5.15	ATP synthase subunit 9
At1g19200	8.27E-04	10.36	Senescence-associated protein
At2g28490	8.78E-04	0.04	Putative seed storage protein (vicilin like)
AtMg00640	9.83E-04	5.88	Plant b subunit of mitochondrial ATP synthase
At2g33380	1.01E-03	5.42	Ca-binding protein, responsive to drought
At5g58570	1.10E-03	4.38	Expressed protein
At3g51860	1.26E-03	5.11	Vacuolar cation/H^+ antiporter
At2g44460	1.51E-03	5.14	Putative beta-glucosidase
At1g12030	1.61E-03	5.59	Hypothetical protein
At1g24575	1.61E-03	0.17	Expressed protein
At1g52690	1.65E-03	3.95	LEA abundant protein
At3g22740	1.81E-03	0.12	Homocysteine S-methyltransferase 3
At1g74890	1.82E-03	0.23	Negative regulator of cytokinin-mediated signals
At1g17960	1.97E-03	4.37	Threonyl tRNA synthetase
At3g03270	2.09E-03	4.03	Universal stress protein/early nodulin protein
At2g07707	2.21E-03	3.63	Hypothetical protein
At3g05400	2.58E-03	3.23	Putative sugar transporter
At1g02850	2.59E-03	6.61	Beta-glucosidase
At1g05680	2.62E-03	4.39	Putative indole-3-acetate beta-glucosyltransferase
At5g13170	2.66E-03	3.37	Senescence-associated protein
At2g26020	2.70E-03	0.11	Pdf1.2b
At1g43910	2.81E-03	3.16	AAA-type ATPase family protein
At2g42540	3.00E-03	4.64	Cold-regulated protein (cor15a), responsive to osmotic stress
At4g38080	3.01E-03	0.07	Proline-rich protein
At5g52300	3.14E-03	5.69	Low-temperature-induced 65-kDa protein
At5g51850	3.32E-03	0.33	Putative protein
AtMg00260	3.39E-03	3.78	Mitochondrial
At1g36180	3.44E-03	5.25	Acetyl CoA carboxylase
At1g26380	3.63E-03	0.10	Putative flavin adenine dinucleotide-linked oxidase
At2g07718	3.68E-03	2.94	Similar to Cyt *b*
At4g11190	3.97E-03	0.25	Putative disease resistance protein
At2g41730	4.05E-03	3.07	Putative protein
At2g42380	4.06E-03	0.11	bZIP TF
At5g44120	4.07E-03	0.03	12S seed storage protein
At2g39850	4.17E-03	0.34	Similarity to subtilisin-like protease
At1g77120	4.21E-03	3.40	Alcohol dehydrogenase, stress responsive
At1g69870	4.31E-03	3.03	Putative peptide transporter
At5g10380	4.37E-03	2.84	Zinc-finger family protein
At3g60140	4.39E-03	10.14	Beta-glucosidase, responsive to starvation
At1g20070	4.39E-03	2.89	Putative protein
At1g23730	4.45E-03	3.01	Putative carbonic anhydrase
At2g43700	4.57E-03	2.69	Lectin protein kinase family protein
AtMg01360	4.63E-03	2.80	Cyt *c* oxidase subunit 1
At2g29490	4.75E-03	3.09	Toxin catabolic process
At2g01210	4.76E-03	0.33	Serine/threonine kinase activity
At2g02390	5.01E-03	2.69	Putative glutathione S-transferase
At4g34135	5.21E-03	3.26	Stress responsive

At5g51190, At1g22810 and At1g28370). These members respond to ethylene and are induced upon cold, drought, osmotic stress and oxidative stress (Nakano et al. 2006a). Genes encoding the WRKY transcription factor family (At1g80840 and At2g46400) were also upregulated in roots by selenate. Similarly, selenate treatment induced expression of *Myb15* (At3g50060), a general stress-responsive transcriptional factor also induced upon cadmium, cold, salt and oxidative stress (Agarwal et al. 2006a). Additional induced transcription factors include the zinc-finger proteins *Zat10* (At1g27730) and *Zat12* (At5g59820).

Induction of general stress proteins included a late embryogenic abundant (LEA) protein (At1g61340) (Mowla et al. 2006), a copper-binding protein (At5g20230) previously reported to be responsive to aluminum and oxidative stress, and members of the small heat shock protein (HSP) family (HSP17.6A, At5g12030 and HSP17.6A-C, At1g59860). HSP17.6A is also responsive to H_2O_2 and overexpression increases drought tolerance (Sun et al. 2001). Lastly, ATP sulfurylase (*APS3* – At4g14680), a key enzyme regulating S and Se assimilation (Pilon-Smits et al. 1999), was induced. This supports previous reports suggesting selenate induces sulfur starvation and activates genes controlling S assimilation (Terry et al. 2000). A Se-induced sulfur starvation response is further suggested by the 30-fold upregulation of a thioglucosidase (At3g60140) thought to break down glucosinolates; these compounds are S-containing secondary metabolites, and their catabolism may recycle a limited pool of S (Dan et al. 2007, Maruyama-Nakashita et al. 2003, 2006).

Shoot tissue

While many stress-responsive transcriptional factors and signaling molecules were identified in the roots, a listing of the 50 most significantly Se-responsive genes in shoot tissue reveals that instead many general stress proteins were upregulated. These include an LEA protein (At1g52690), *Cor15a* (At2g42540) – a cold-regulated protein responsive to osmotic stress, a low-temperature induced protein (At5g52300), a glutathione transferase (At2g02390) and several disease- and stress-induced proteins (At4g11190, At3g03270 and At4g34135). Also of interest in this group are four mitochondrial-encoded transcripts (AtMG00040, AtMG00640, AtMG00260 and AtMG01360) that encode subunits of ATP synthase and the electron transport chain. These proteins may be upregulated in response to perceived starvation or a shortage of photosynthesis-derived assimilates fueling ATP production. Other nuclear-encoded respiratory proteins include acetyl CoA carboxylase (At1g36180) and an electron transport protein similar to Cyt *b* (At2g07718). Further evidence of sugar utilization includes upregulation of a carbohydrate transporter (At3g05400), a galactinol synthase (At1g56600) reported to be responsive to drought and cold and a glycosyl hydrolase (At1g02850) involved in carbohydrate catabolism. Thirteen genes were found to be downregulated in shoots, including two seed-storage proteins (At2g28490 and At5g44120). These proteins have nutrient reservoir activity, and their downregulation further points to a reshuffling of metabolites during starvation. Intriguingly, also downregulated in shoot tissue was Pdf1.2b, a well-known plant defensin (Pdf) regulated by JA and ethylene (Penninckx et al. 1998) and induced by various biotic and abiotic stresses.

Shoot-tissue transcripts responded differently to selenate than those in the roots. Genes induced by abiotic stress could be divided into two groups: regulatory proteins (transcriptional factors and kinases), which are typical of Se responses in root tissue, and functional proteins, which in this study were generally associated with shoot tissue responses.

We considered it worthy to look beyond the 50 most significantly Se-responsive genes and explore the nearly 900 root and 400 shoot Se-responsive transcripts identified (*Supplementary material* Table S1). This approach would allow us to develop a more complete understanding of how Se stress is perceived by the plant after 10 days of selenate treatment, how this stress changes gene regulation and the key pathways involved in mediating a response to Se. Additionally, we were interested in determining if genes categorized by biological processes were overrepresented in our data set of Se-responsive genes in root or shoot tissue. Using the list of differentially expressed genes (roots and shoots separately), Gene Ontology (GO) Biological Processes terms were tested for overrepresentation using the hypergeometric test. GO terms associated with *P* values of 0.01 or less are shown in *Supplementary material* (Table S2). Genes categorized as responsive to abiotic stress (heat, salt, drought and osmolarity) and involved in S utilization, amino acid biosynthesis and hormone (ethylene and JA) signaling and synthesis were all significantly overrepresented ($P < 0.001$) in the microarray data (*Supplementary material* Table S2). Using these overrepresented groups as a guide, selected Se-responsive genes of interest in root and shoot tissues were then categorized into families or biological functions (Table 3).

Selenate upregulates transcripts involved in JA and ethylene synthesis, as well as transcriptional factors responsive to these two stress hormones

JA and ethylene are considered as stress-responsive hormones and are capable of eliciting a defense response. Six genes involved in the synthesis of JA were found to be upregulated by selenate in root tissue, including two lipoxygenases (At1g72520 and At3g25780), which had a fold change of 84 and 116, respectively, compared with plants on control media. Two genes encoding allene oxide cyclase (At3g25760 and At1g17420), downstream of the lipoxygenases in JA synthesis, were also upregulated by Se. Microarray analysis also suggests that Se treatment induces the synthesis of ethylene. ACC synthase, the key step in ethylene production, was

Table 3. Selected selenate-responsive genes were categorized by function or gene family.

Locus	Tissue	*P* value	Fold change	Annotation
Ethylene synthesis				
At4g11280	r	3.36E-05	16.54	ACC synthase
At3g61400	r	4.29E-04	4.12	1-aminocyclopropane-1-carboxylate oxidase
At4g26200	s	0.012	2.43	1-aminocyclopropane-1-carboxylate synthase (ACS7)
At5g20400	s	0.028	2.53	Ethylene-forming-enzyme-like dioxygenase-like protein
ERF transcription factor family (DREB, CBF, AP2)				
At2g44840	r	1.23E-05	86.05	Ethylene response element-binding protein (AtERF13)
At1g74930	r	1.42E-05	48.16	Member of the DREB subfamily A-5 of ERF/AP2
At1g19210	r	1.59E-05	34.55	Member of the DREB subfamily A-5 of ERF/AP2
At4g34410	r	1.61E-05	263.44	Member of the ERF subfamily B-3 of ERF/AP2
At5g51190	r	3.33E-05	14.97	Member of the ERF subfamily B-3 of ERF/AP2
At1g22810	r	5.96E-05	9.06	Member of the DREB subfamily A-5 of ERF/AP2
At5g52020	r	8.05E-05	13.62	Member of the DREB subfamily A-4 of ERF/AP2
At1g28370	r	1.03E-04	14.74	AtERF11/CEJ1
At4g17490	r	1.10E-04	9.2	Ethylene-responsive element-binding factor-like protein (AtERF6)
At4g17500	r	1.30E-04	5.87	Ethylene-responsive element-binding factor (AtERF1)
At5g44350	r	1.43E-04	4.83	Ethylene-regulated nuclear protein ethylene responsive transcriptional factor 2-like protein
At2g31230	r	1.90E-04	4.35	Ethylene response factor (AtERF15)
At1g21910	r	2.00E-04	6.16	Member of the DREB subfamily A-5 of ERF/AP2
At1g12610	r	2.06E-04	227.79	Member of the DREB subfamily A-1 of ERF/AP2 (DDF1)
At1g06160	r	2.46E-04	7.12	Ethylene response factor (AtERF94)
At5g61590	r	2.92E-04	0.23	Ethylene-responsive element-binding factor (AtERF107)
At5g47230	r	3.89E-04	4.5	Ethylene-responsive element-binding factor 5 (AtERF5)
At1g77640	r	5.97E-04	4.46	Member of the DREB subfamily A-5 of ERF/AP2 (AtERF13)
At3g15210	r	6.64E-04	3.12	Ethylene-responsive element-binding factor 4 (AtERF4)
At5g47220	r	1.03E-03	21.38	Ethylene-responsive element-binding factor 2 (AtERF2)
At5g05410	r	1.12E-03	3.79	DREB2A
At3g11020	r	2.14E-03	2.18	DREB2B transcription factor
At3g24500	r	2.24E-03	2.05	Ethylene-responsive transcriptional coactivator
At4g25470	r	2.99E-03	17.17	Member of the DREB subfamily A-1 of ERF/AP2 (DREB1c/CBF2)
At5g13330	s	8.23E-03	2.78	Member of the ERF subfamily B-4 of ERF/AP2
At1g44830	r	0.015	2.26	Member of the DREB subfamily A-5 of ERF/AP2
At4g25480	r	0.028	2.05	Member of the DREB subfamily A-1 (DREB1a/CBF3)
At3g23240	s	0.048	0.46	ERF1
JA synthesis				
At1g72520	r	1.44E-05	84.25	Lipoxygenase
At3g25760	r	5.13E-04	3.29	Allene oxide cyclase (JA), AOC1
At3g25780	r	5.45E-04	7.08	Allene oxide cyclase (JA synthesis), AOC3
At1g17420	r	6.54E-04	116.69	Lipoxygenase (LOX3)
At5g42650	r	8.58E-04	7.39	Allene oxide synthase (AOS/CYP74A)
At2g06050	r	1.05E-03	4.19	12-oxophytodienoate-10,11-reductase, OPR3
Auxin-regulated/growth hormones				
At2g45210	r	1.36E-03	0.36	Putative auxin-regulated protein
At2g33830	r	1.60E-03	0.31	Dormancy/auxin-regulated protein
At2g46690	r	1.89E-03	0.34	Putative auxin-regulated protein
At1g59500	r	2.76E-03	0.46	Auxin-regulated protein GH3
At1g60750	r	3.53E-03	2.87	Auxin-induced protein
At3g47620	r	3.52E-03	0.45	Putative auxin-induced bHLH (helix-loop-helix) transcription factor
At3g61900	r	0.021	0.42	Putative protein auxin-induced protein
At3g03830	s	0.013	0.43	Putative auxin-induced protein
At1g73590	s	0.014	0.45	Auxin efflux carrier protein (PIN1)
At1g62500	s	0.022	0.45	Auxin-responsive proline-rich seed storage/lipid transfer protein
At3g63440	s	0.029	0.11	Cytokinin oxidase (ATCKX6)

Table 3. Continued.

Locus	Tissue	*P* value	Fold change	Annotation
Sulfur metabolism				
At4g14680	r	1.31E-04	5.91	APS3
At4g08620	r	1.82E-04	10.42	Sulfate transporter SULTR1;1
At5g43780	r	2.07E-04	0.25	APS4
At5g10180	r	3.10E-04	6.44	Sulfate transporter (SULTR2;2)
At4g04610	r	4.24E-04	3.13	5′-adenylylsulfate reductase (APR1)
At4g21990	r	5.02E-04	5.12	5′-adenylylsulfate reductase (APR3)
At1g52820	r	5.81E-04	0.06	Similar to AOP1-glucosinolate synthesis
At1g62180	r	2.14E-03	2.58	5′-adenylylphosphosulfate reductase (APR2)
At2g17640	r	2.76E-03	2.12	serine acetyl transferase
At1g80310	r	5.50E-03	0.46	Putative sulfate transporter
Glucosinolate biosynthesis and breakdown				
At3g60140	r	9.12E-06	30.76	Beta-glucosidase
At2g44460	r	1.19E-04	252.1	Beta-glucosidase
At5g23010	s	1.31E-02	0.45	Methylthioalkylmalate (MAM1)
At1g16410	s	1.67E-02	0.5	Cyt P450 CYP79F1; glucosinolate synthesis
At5g28520	r	4.52E-02	0.15	Myrosinase-binding protein
At3g19710	s	5.26E-03	0.36	Branched-chain amino acid aminotransferase (BRAT4)
At3g19710	r	5.57E-03	0.48	Branched-chain amino acid aminotransferase (BRAT4)
Proline/cell wall synthesis				
At4g02270	r	2.40E-04	0.27	Extensin-like protein
At5g04960	r	2.57E-04	0.28	Pectinesterase
At5g35190	r	2.80E-04	0.25	Extensin-like protein extensin
At3g62680	r	4.19E-04	0.33	Proline-rich cell wall protein
At2g45220	r	4.51E-04	0.3	Putative pectinesterase
At4g34150	r	5.70E-04	3.55	Hydroxyproline-rich glycoprotein – responsive to cold
At4g12550	r	7.12E-04	0.36	Putative cell wall–plasma membrane-disconnecting protein
At1g12040	r	1.10E-03	0.37	Putative extensin
At3g50630	r	1.32E-03	0.44	Putative protein cyclin-dependent kinase inhibitor (ICK1)
At5g19800	r	3.72E-03	0.29	Proline-rich protein extensins
At3g10710	r	3.91E-03	0.22	Putative pectinesterase
At1g62510	r	4.20E-03	0.48	Proline-rich lipid transfer protein
At5g07570	r	5.08E-03	0.48	Glycine/proline-rich protein
At1g76310	s	5.09E-03	0.37	Similar to B-like cyclin
At2g33790	r	6.50E-03	0.5	Putative proline-rich protein
At3g54580	r	8.65E-03	0.36	Extensin precursor-like protein
At3g54590	r	9.77E-03	0.47	Extensin precursor-like protein
At5g13840	s	0.011	0.4	Cell cycle switch protein
At1g54970	r	0.012	0.49	Proline-rich protein
At5g38710	s	0.013	0.34	Proline oxidase
At2g24980	r	0.015	0.4	Proline-rich cell wall protein
At4g03270	s	0.017	0.28	Putative D-type cyclin
At4g30140	s	0.018	0.29	Putative proline-rich protein
At1g62500	s	0.022	0.45	Putative proline-rich cell wall protein
At2g17620	s	0.025	0.49	Putative cyclin 2
At2g46630	s	0.028	0.46	Putative extensin
At4g13390	r	0.037	0.14	Extensin-like protein extensin class 1 protein
Stress-responsive transcription factors				
At1g80840	r	4.88E-06	59.17	WRKY40 – defense related
At2g46400	r	1.15E-05	25.76	WRKY46
At1g27730	r	1.31E-05	29.25	Salt-tolerance zinc-finger protein (ZAT10)
At5g59820	r	6.38E-05	7.55	Zinc-finger protein (Zat12) signaling of ROS
At5g04340	r	1.60E-04	4.44	zat6
At5g49520	r	3.97E-04	5.82	WRKY48
At2g38470	r	6.39E-04	4.24	WRKY33

Table 3. Continued.

At3g53600	r	6.78E-03	32.55	zinc finger-like protein (Zat11)
At2g30250	s	0.042	2.01	WRKY25
HSP				
At5g12030	r	4.93E-05	21.56	HSP17.4-C1
At1g59860	r	8.09E-05	10.1	HSP17.6A-C1
At1g53540	r	9.56E-05	7.61	HSP17.6C-C1
At5g12020	r	1.49E-03	43.21	HSP17.6-CII
At2g26150	r	1.57E-02	21.58	Heat shock transcription factor (HSFA2)
At3g46230	r	1.34E-04	16.67	HSP17.6-CII
At5g52640	r	1.86E-04	4.6	HSP81-1
At3g12580	r	2.27E-04	3.96	HSP70
At5g51440	r	1.00E-03	3.04	Mitochondrial HSP23.5
At4g18880	r	2.82E-03	2.18	Heat shock transcription factor (HsfA4a/HSF21)
At1g54050	r	3.60E-03	3.14	HSP17.4-CIII
At2g20560	r	4.37E-03	2.05	DNAJ HSP family
At1g74310	r	4.98E-03	2.38	HSP101
At5g43840	s	0.026	10.64	Heat shock transcription factor (HSFA6A)
At5g12030	s	0.026	2.88	HSP17.7-CII
At5g51440	s	0.047	2.03	Mitochondria HSP-22
Amino acid metabolism				
At2g02010	r	1.05E-04	11.57	Putative glutamate decarboxylase
At3g56200	r	2.53E-04	3.59	Amino acid transporter
At2g24850	r	3.36E-04	4.75	Putative tyrosine aminotransferase
At1g36370	r	7.30E-04	2.97	Serine hydroxymethyltransferase
At3g08860	r	1.35E-03	4.8	Putative beta-alanine-pyruvate aminotransferase
At2g38400	r	1.41E-03	0.28	Putative beta-alanine-pyruvate aminotransferase
At1g17960	s	1.97E-03	4.37	Threonyl tRNA synthetase
At3g23250	r	2.18E-03	135.84	myb15 – activates shikimate pathway
At2g13360	r	2.18E-03	0.41	Alanine glyoxylate aminotransferase
At2g17640	r	2.76E-03	2.12	Serine acetyl transferase
At3g19710	s	5.26E-03	0.36	Branched-chain amino acid aminotransferase (BRAT4)
At3g19710	r	5.57E-03	0.48	Branched-chain amino acid aminotransferase (BRAT4)
At3g47340	s	7.07E-03	0.37	Glutamine-dependent asparagine synthetase
At3g61300	r	0.012	0.44	Tyrosine decarboxylase
At1g08090	r	0.013	3.71	High-affinity nitrate transporter NRT2
At5g16570	s	0.013	0.37	Putative glutamine synthase
At5g38710	s	0.013	0.34	Proline oxidase
At3g08860	s	0.014	2.07	Putative beta-alanine-pyruvate aminotransferase
At4g23600	s	0.015	2.33	Tyrosine transaminase
At5g19530	s	0.020	0.41	Spermine synthase (ACL5)
At1g59740	s	0.021	0.5	Oligopeptide transporter
At4g28680	s	0.024	0.46	Tyrosine decarboxylase
At2g13810	r	0.024	0.49	Putative aspartate aminotransferase

upregulated 16-fold in roots. ACC is converted into ethylene by aminocyclopropane-1-carboxylate oxidase; this gene was upregulated four-fold in roots. Two other genes involved in ethylene formation were upregulated in shoots (Table 3).

The Arabidopsis genome consists of over 1600 predicted transcriptional factors, representing 6% of the total genome. The ethylene signaling pathway involves EIN1, EIN2 and EIN3 (Benavente and Alonso 2006), which can induce downstream ERF transcriptional factors. The ERF family, including dehydration responsive element binding-protein (DREB), cold-binding factor (CBF) and AP-binding elements, contains roughly 120 transcriptional factors (Nakano et al. 2006a). Treatment with selenate affected expression of 27 ERF genes, all of which were upregulated except AtERF107 (At5g61590) and AtERF92/*ERF1* (At3g23240). AtERF11/*CEJ1* was upregulated 15-fold by Se. This gene has been found to be cooperatively regulated by ethylene and jasmonate (Nakano et al. 2006b). Of the 27 ERF genes upregulated by Se, only two were identified in shoot tissue: AtERF113 (At5g13330) and (again) *ERF1*. The fact that *ERF1* is downregulated in the shoots is intriguing because it also regulates many genes

responsive to ethylene and JA (Lorenzo et al. 2003). The ERF family responds to various abiotic stresses, and their regulation is not unique to Se. Many of these Se-responsive ERF genes have previously been overexpressed, and the increased tolerance of the overexpressors to various abiotic stresses has been reviewed (Agarwal et al. 2006b, Nakano et al. 2006a). For example, *Cbf1* overexpression increased tolerance to drought, salt and cold and induced expression of other stress-responsive genes. (Jaglo-Ottosen et al. 1998). In this study, Cbf1 was upregulated 227× in root tissue when plants were grown on Se.

Other transcriptional factors that were upregulated by Se treatment belong to the Myb, WRKY, Zinc transporter in Arabidopsis thaliana (ZAT) and heat shock factor (HSF) families and have a known role in responding to abiotic stress. Myb2, 15 and 77 were upregulated by Se; these transcripts respond very strongly to ethylene, and to a lower extent to JA, but not to the plant hormones salicyclic acid (SA), GA and ABA (Yanhui, et al. 2006). Similarly, the WRKY transcriptional factors (TFs) identified in this study (WRKY33, 40, 46 and 48) are also known to respond to ethylene (Xu et al. 2006), further pointing to a role for ethylene and JA in eliciting a defense response to Se. Furthermore, recent evidence suggests that *Zat12*, *HSFA2* and *HSFA6a* (all of which are upregulated by Se) sense H_2O_2, implicating their role in reactive oxygen signaling (Davletova et al. 2005, Miller and Mittler 2006).

The similarity between transcriptional factors responsive to selenate and those responsive to other abiotic stresses compelled us to use the AtGenExpress project to identify overlap in transcriptome changes in response to selenate, salt and osmotic stress (Kilian et al. 2007). For root and shoot tissue, roughly 62% of the Se-responsive genes were also differentially regulated during salt and/or osmotic stress (Fig. 2). Despite the fact that these comparisons suggest that commonality exists during the response to abiotic stresses, it is noted that these microarray experiments differed in the length of treatment and media composition. For example, our study used 1% sucrose while the salt and osmotic stress experiments used 0.5% sucrose.

Mutant analysis confirms a role for ethylene and JA in Se tolerance

A total of 27 ethylene-responsive transcriptional factors were observed to be differentially regulated in response to Se. We deemed it important to determine the role of ethylene in Se tolerance. Considering the size of the ERF family and the likelihood of redundancy, we targeted the ethylene pathway upstream of the ethylene-responsive transcriptional factors. Thus, Arabidopsis mutants (ecotype Columbia) with defects in ethylene synthesis and signaling were analyzed for Se tolerance. Arabidopsis plants containing a T-DNA insertion in ACC synthase (ACS6) were grown with or without 40 μM selenate. In view of a small difference in root length between WT and ACS6 knockout plants (*acs6*) when grown on control media, selenate tolerance was calculated as selenate tolerance index (growth on Se/growth on control). It appears that knockout of ACS6 decreases tolerance to selenate, as judged from root growth (Fig. 3). Similarly, mutant plants defective for upstream ethylene signaling genes (*ein2-1* and *ein3-1*) were both less tolerant to selenate than WT plants (Fig. 3). Furthermore, plants overexpressing ERF1 exhibited an increase in selenate tolerance, which makes the downregulation of ERF1 in shoot tissue intriguing. Lastly, *jar1-1* (jasmonic acid resistant, involved in JA signaling) mutant plants showed a decreased tolerance to Se. It should be noted that *ein2-1*, *ein3-1* and *jar1-1* were not induced by Se. Still the results from these genetic studies are in agreement with those from the gene expression studies, which indicate

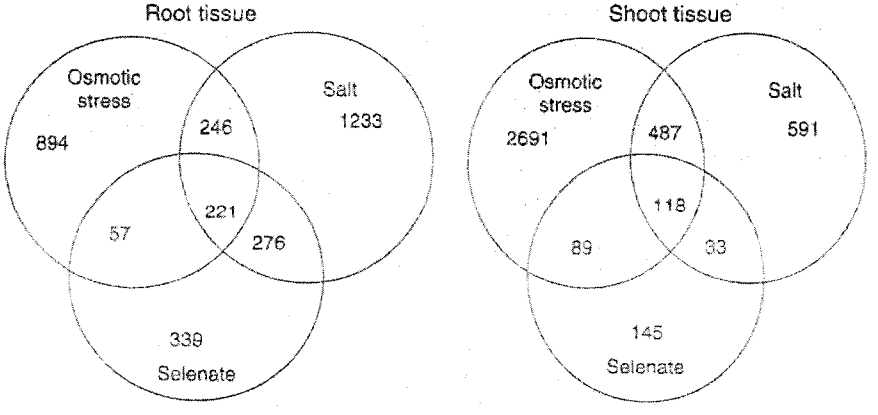

Fig. 2. Venn diagram showing differentially regulated genes in response to selenate, osmotic stress and salt stress in root and shoot tissues.

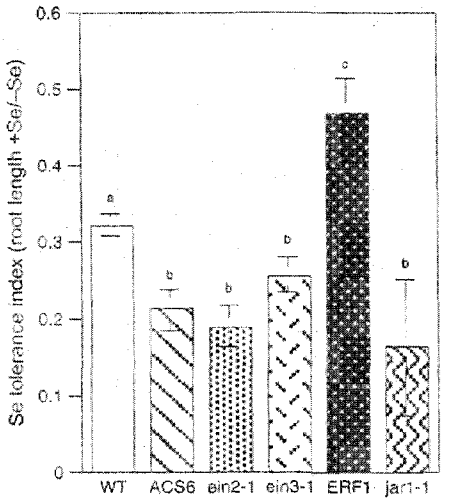

Fig. 3. Selenate tolerance index (root length on selenate/root length on control medium) for WT plants and mutant and transgenic seedlings (*acs6*, *ein2-1*, *ein3-1* and *jar1-1* and an overexpressor of ERF1) grown for 10 days on control medium and on medium with selenate. Shown are the means ($n = 30$) and SE of the mean. Lowercase letters above bars denote significant differences ($P < 0.05$).

that both ethylene and JA play important roles in the defense response to selenate.

Selenate upregulates the sulfur assimilation pathway and changes metabolite abundance

Previous studies have shown that selenate mimics S starvation (Takahashi et al. 2000). Under selenate treatment, transcripts encoding sulfate transporters SULTR1;1 and SULTR2;1 were increased in root tissue, suggesting S deficiency. These transporters, whose mRNA levels also increase during S deficiency, are thought to be responsible for transporting sulfate into the root cells and for loading sulfate into the root vascular tissue, respectively. Several genes involved in S assimilation were also upregulated, including an APS3 and three adenosine-phosphosulfate reductase genes (APR1, 2 and 3).

Further evidence that the plants perceive sulfur limitation in the presence of Se is seen in the abundance of transcripts regulating the synthesis of S-rich glucosinolates. The pathways for glucosinolate synthesis and degradation are suggested to be regulated coordinately with sulfur assimilation (Maruyama-Nakashita et al. 2003, 2006). Upregulation of two genes in root tissue encoding thioglucosidases (At2g44460, 252-fold; At3g60140, 31-fold) was observed under selenate stress; these enzymes are capable of breaking down glucosinolates in order to recycle S (Dan et al. 2007). *BRAT4*, a branched-chain aminotransferase, was downregulated both in root and shoot tissue, as was *MAM1* in shoot tissue. *BRAT4* and *MAM1* participate in the methionine-derived glucosinolate biosynthesis in Arabidopsis; expression of *BRAT4* and *MAM1* increase in response to wounding (Schuster et al. 2006). Furthermore, a Cyt P450 CYP79F1 for methionine-derived glucosinolate synthesis was downregulated in shoot tissue under Se stress, and a myrosinase-binding protein in root tissue was also downregulated.

As already stated, analysis of the microarray data indicates that Se treatment induces S deficiency. Previously, it was reported that total S content increased 1.5-fold in shoots, and marginally decreased in roots, in plants grown on the same Se treatment used in this study (Van Hoewyk et al. 2005). We performed a more detailed analysis of S metabolites to gain a better understanding how Se affects S metabolites. When plants were grown on selenate, sulfate increased five-fold compared with control media (Fig. 4A). This result is in agreement with the microarray data that indicated upregulation of sulfate transporters. In addition to sulfate, reduced organic S metabolites compose a large fraction of the S pool. To determine if levels of reduced organic S metabolites are affected by selenate treatment, the levels of non-protein thiols were measured in shoot tissue. Non-protein thiol levels were nearly three-fold lower when grown on Se (Fig. 4B). A large fraction of the non-protein thiol pool consists of glutathione, which is involved in regulating the cell's redox state as well as the storage and transport of reduced sulfur in plants. While the level of GSSG did not change during Se treatment, the reduced form GSH, which represents a majority of the glutathione pool, declined four-fold (Fig. 4C, F). Glutathione is a tripeptide containing the amino acids glutamine, glutamate and cysteine. Levels of GSH decreased perhaps in an effort to conserve more important primary S compounds, such as S-containing proteins. An elemental analysis of total protein in the shoots of Arabidopsis plants grown with and without Se indicates that S in protein decreased during Se treatment (Fig. 4D). Taken together, decreased levels of non-protein thiols, GSH and S in protein suggest that Se treatment induced S starvation, which likely caused the upregulation of genes involved in sulfate transport and resulted in the noticeable increase in sulfate content.

The observed decrease of S in proteins prompted the investigation of whether or not the levels of the S amino acids methionine and cysteine were affected in plants grown on Se. The abundance of these two amino acids decreased in shoots of Se-treated plants (Table 4), in agreement with the earlier observation that other organic S compounds decreased upon selenate treatment; however, this decrease was non-significant. Aspartic acid and glutamic acid both also decreased in shoot tissue when plants were grown on selenate. In contrast, selenate treatment significantly

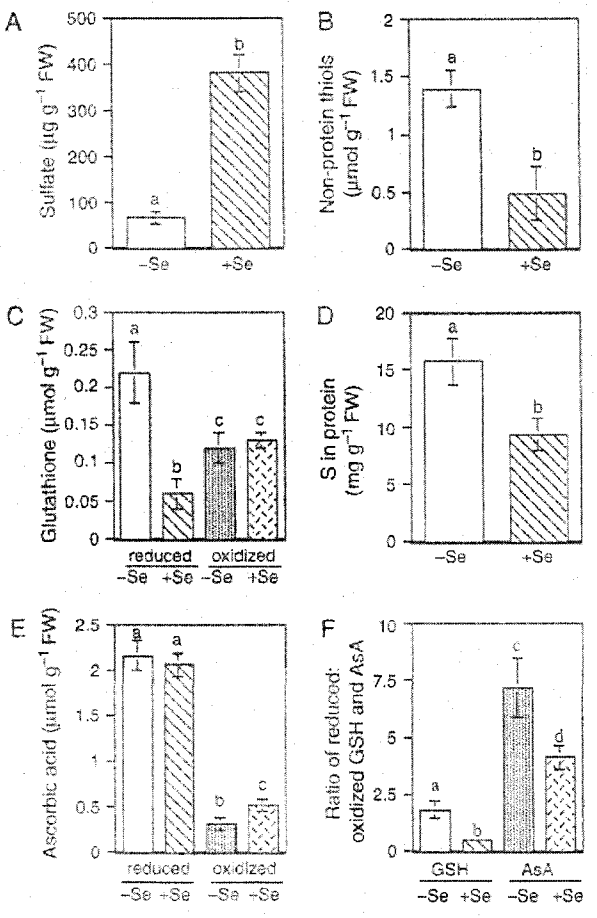

Fig. 4. Shoot tissue of seedlings grown for 10 days on control media (−Se) and on media with selenate (+Se) were analyzed for (A) sulfate, (B) non-protein thiols, (C) GSH and GSSG, (D) amount of S in protein, (E) reduced and oxidized ascorbic acid and (F) the ratio of GSH and GSSG and AsA, which is indicative of the redox state in plants. Shown are the means (n = 3) and se of the mean. Lowercase letters above bars denote significant differences ($P < 0.05$).

increased levels of asparagine, serine, glycine, glutamine and arginine in shoot tissue compared with plants grown on control media (Table 4). Overall, the response to selenate stress was an increase in total amino acid content in shoot tissue. This may reflect increased levels of JA in Se-treated plants. Previously, *Medicago truncatula* plants treated with methyl JA showed an increase in amino acid content, and it was speculated that carbohydrate metabolites were being reallocated into amino acids (Broeckling et al. 2005). Although sugar metabolites were not measured in this study, an increase in amino acid content may reflect a role in the defense response for some amino acids. Alternatively, if Se reduces plant growth, a decreased demand for proteins could feasibly result in temporary amino acid accumulation.

Table 4. Amino acid and inorganic nitrogen content (pmol/mg FW) in shoot tissue of plants grown on control media or selenate for 10 days. Shown are the mean (n = 3) and se in parentheses. *A significant increase ($P < 0.05$) in amino acid or inorganic nitrogen content between the two treatments.

ala	108 (15.2)	88 (18)
asn	318 (34)	502 (54)*
arg	58 (3.6)	92 (9.6)*
asp	94 (16.2)*	30.6 (5.0)
cys	2.6 (0.2)	2.2 (0.2)
gln	4258 (662)	8376 (1308)*
glu	109 (9.4)*	40 (5.2)
gly	210.6 (52)	712 (138)*
his	10.8 (1.6)	16.6 (2.2)
ile	3.2 (0.6)	3.2 (0.8)
leu	4.2 (0.8)	3.2 (0.6)
lys	5.2 (1.0)	5.0 (1.1)
met	2.6 (0.6)	1.4 (0.2)
phe	4.8 (0.6)	3.2 (0.4)
pro	212.6 (19.8)	166 (22)
ser	152 (18)	400 (61)*
thr	60 (6.8)	80 (12.2)
tyr	3.2 (0.4)	2.8 (0.4)
val	11.2 (1.4)	11.4 (1.2)
NH_3	97 (8.8)	86 (3.6)
NO_3	3614 (147)*	1879 (103)

The general increase in amino acid content in Se-treated plants is perhaps more difficult to explain than the decrease in cysteine and methionine. While there was an increase (3.7-fold) of a high-affinity nitrate transporter (Table 3), there was a decrease in both nitrate and ammonia in shoots of plants grown on Se (Table 4). Also noteworthy in Se-treated plants is an (non-significant) increase in the levels of the aromatic amino acids tyrosine and phenylalanine, which may ultimately lead to protective flavonoids. Myb15, a transcriptional factor upregulated 130-fold in Se-treated root tissue, is known to activate the shikimate pathway (tyrosine and phenylalanine synthesis) in plants (Chen et al. 2006).

As previously mentioned, reduced GSH declined nearly four-fold in the shoots of Se-treated plants, while oxidized GSH levels were unchanged. GSH and AsA participate in the antioxidant defense system. The ratio of reduced AsA and its oxidized form, dehydroascorbic acid (DHA), has been used to gauge the redox state in plants (Yoshida et al. 2006). While levels of AsA were hardly affected by Se, DHA significantly increased (Fig. 4E, F). Thus, in Se-treated plants, the AsA/DHA and GSH/GSSG ratios both decreased, suggesting Se-related oxidative stress.

Selenate treatment downregulates transcripts involved in growth and development

Selenate treatment reduced the length of roots nearly three-fold, as well as the mass of shoot tissue

(*Supplementary material* Fig. S1). The reduced biomass of Arabidopsis on selenate may reflect changes in expression levels of transcripts regulating growth and development. Indeed, five genes encoding proteins known to control the progression of the cell cycle (e.g. cyclins) were found to be downregulated, of which four were in shoot tissue (Table 3). Recently, it was discovered that Arabidopsis plants treated with cadmium showed downregulation of genes involved in cell wall synthesis (Herbette et al. 2006). In this study, selenate induced the downregulation of five transcripts encoding proline-rich cell wall proteins, three pectinesterases and a cell wall-dissociating factor. Yet in contrast to the cadmium study, which showed upregulation of cell wall extensins, selenate repressed eight extensins. Up to 30% of photosynthate can be directed to the cellulose-containing cell wall. Together, these data suggest that cell growth and division are being repressed in response to selenate stress.

Next, Se-responsive genes in root and shoot tissues were screened to identify if selenate affects hormones associated with plant growth and development. Among its myriad of functions in plant biology, auxin is best known to induce growth. Auxin was recently shown to suppress a thioglucosidase (At2g44460), and it was speculated that auxin may have a role in negatively regulating the −S response (Dan et al. 2007). In this study, the thioglucosidase was upregulated 252-fold by Se in root tissue. Although −S conditions do not decrease auxin levels (Kutz et al. 2002), it is instead speculated that auxin sensitivity is decreased during S starvation (Dan et al. 2007). Indeed, *NIT3*, one of four nitrilases that forms IAA, was upregulated in both root and shoot tissues by Se and was previously discovered to be upregulated in response to −S and involved in glucosinolate catabolism (Kutz et al. 2002). In our selenate study, 10 transcripts encoding auxin-responsive proteins were identified to be affected by Se, 9 of which were downregulated. These data suggest that selenate may also reduce auxin sensitivity.

While auxin is believed to play a role in the release of thiol groups from S storage sources during S starvation (Dan et al. 2007), it is not thought to be the key hormone regulating the transport of S into the roots. Instead, cytokinin is suspected of controlling S uptake. Recent evidence suggests that the hormone cytokinin decreases sulfate uptake into roots, as explained by the concomitant decrease in the S transporters SULTR1;2 and SULTR1;1 (Maruyama-Nakashita et al. 2004). Although cytokinin represses root development, it likely has an antagonistic effect in shoot development (Werner et al. 2003). In this study, Se treatment decreased the transcript levels of cytokinin oxidase (AtCKX6 and At3g63440) nearly 10-fold in shoot tissue. There are seven cytokinin oxidases in Arabidopsis that catalyze the irreversible degradation of the hormone cytokinin. Overexpression of At3g63440, which is primarily localized in the leaf vasculature, showed cytokinin deficiency symptoms (Werner et al. 2003). Interestingly, cytokinin was shown to increase transcript levels of APR1, a key enzyme in S reduction (Ohkama et al. 2002). Thus, downregulation of AtCKX6 suggests that levels of cytokinin may increase in leaf tissue when plants are grown on Se, although this remains to be determined.

Two stress-responsive Pdfs are downregulated by Se in shoots

Se may cause toxicity by (1) inducing S starvation and thereby decreasing the S pool, (2) competing with S for biochemical functions (proteins, secondary metabolites, Fe–S clusters, etc.) or (3) innately triggering stress, e.g. by creating free radicals or via another, unknown mechanism. The observation that both the AsA and glutathione pools were in a more oxidized state may indicate that indeed free radicals also were a factor in the plants' Se response. The results from this study also are in agreement with previous research in suggesting that Se induces S starvation, resulting in upregulation of genes involved in S transport and assimilation. Sulfate transport appeared to be more upregulated than sulfate assimilation, judged from the observed accumulation of sulfate and the decrease in levels of organic S compounds. It is intuitive that S starvation should decrease secondary S metabolites, and data are presented to suggest that levels of glucosinolates and non-protein thiols have decreased. Accordingly, this should hold true for other S-containing molecules and may partly explain the observation that transcript levels encoding the stress proteins Pdf1.2a (At5g44420) and Pdf1.2b (At2g26020) decreased four-fold and 10-fold, respectively, in shoot tissue. Both of these proteins are Pdfs and universal stress proteins and as such are upregulated under various biotic and abiotic stresses; furthermore, *Pdf1.2* transcripts respond to the plant hormones ethylene and JA (Larsen and Cancel 2004). However, both proteins are cysteine rich (10% cysteines), and their downregulation may be explained by (1) S starvation or (2) the relatively high chance for Se-cysteine incorporation.

Another intriguing possibility to explain the decrease in *Pdf1.2a* and *Pdf1.2b* transcripts in shoot tissue is that the stress signaling pathway perceived in the roots does not reach or affect the shoots. Transcripts governing the synthesis of and responses to ethylene and JA were for the most part only upregulated in roots. Ethylene and JA pathways are synergistic and converge at the transcriptional activation of *ERF1* (Lorenzo et al. 2003), which was downregulated by Se in shoots, and *CEJ1*, which was

upregulated in roots. *Pdf1.2a* and *Pdf1.2b* respond to ethylene and JA and were upregulated in plants overexpressing ERF1 (Berrocal-Lobo et al. 2002). Therefore, it remains a possibility that in shoots, ethylene and JA signaling are not contributing forces in mounting a defense against selenate.

Macroarray analysis confirms transcript fold changes in microarray study

A potential concern of the microarray study performed was the use of only two biological replicates. Although the AtGenExpress abiotic stress expression data set also used two replicates (Kilian et al. 2007), the need to validate our microarray data by another method was paramount to our interpretation of the data. To date, quantitative real-time polymerase chain reaction (qRT-PCR) has been used to help confirm the expression of selected genes identified in a microarray analysis. Here, we performed two replicates of cDNA macroarray experiments to help substantiate the reliability of the microarray analysis. Thirty-one Se-responsive genes representing a range of fold changes were selected from the microarray analysis and compared with a macroarray analysis. The fold change of the selected genes was often higher in the microarray analysis compared with the macroarray. The opposite effect has been noted when comparing estimated fold change from microarray analysis to qRT-PCR (Dallas et al. 2005) or when using spiked-in transcripts (Choe et al. 2005). Nonetheless, a regression analysis reveals that the fold changes in the selected genes between the two experiments show a significant (P value <0.0001) and positive correlation ($R = 0.72$). A comparison of the microarray and macroarray analysis for all 31 selected genes is provided in *Supplementary material* (Table S3). A comparison of these two analyses suggests that macroarray analysis is a robust method for validating microarray data. This technique is relatively easy to use and not as cost prohibitive as qRT-PCR.

Concluding remarks and future direction

As suggested by the microarray analysis, Se increased sulfate levels, but this did not translate into higher levels of other S metabolites. This suggests that in response to Se stress, one or more enzymes involved in sulfate assimilation was limiting the synthesis of more reduced S metabolites. Previous attempts to augment Se accumulation and tolerance in plants have heavily focused on manipulation of the genes involved in S transport and assimilation and have shown the potential of overexpressing S assimilation enzymes for enhancing Se tolerance, accumulation and volatilization. Our goal in this study was to better understand how Se modulates gene expression and to identify additional Se-responsive genes using a transcriptomics approach and to use this data as a tool to pinpoint key genes that may increase Se tolerance in plants. Microarray analysis indicated that ethylene and JA are the key hormones regulating a defense response to Se and that the hormones ABA and salicylic acid have little effect. We focused our efforts on determining if ethylene and JA had an effect on Se and found that Arabidopsis mutant plants defective in the synthesis or signaling of these two hormones were less tolerant to selenate and that hormone overproduction increased Se tolerance. Thus, future genetic engineering approaches may focus on controlled overproduction of these hormones. Successful strategies for increasing Se tolerance in plants are likely not limited to manipulating genes governing S metabolism and ethylene and JA synthesis. Future research efforts may also include overexpression of other Se-responsive genes identified in this study. Understanding the molecular responses to selenate and further characterization of Se-responsive genes identified in this study may help create plants that can better tolerate and accumulate Se in order to achieve the goals desired for effective phytoremediation.

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Supplementary material

The following supplementary material is available for this article:

Fig. S1. Se treatment reduced the root length of Arabidopsis plants after 10 days of growth.

Table S1. List of all the Se-responsive genes in root tissue (n = 893) and shoot tissue (n = 385) using the minimal criteria of P value <0.05 and fold change >2 or <0.5.

Table S2. Se-responsive genes in root and shoot tissues were categorized into biological processes and a list of overrepresented groups is presented.

Table S3. A comparison of the microarray and macroarray analysis for all 31 selected genes.

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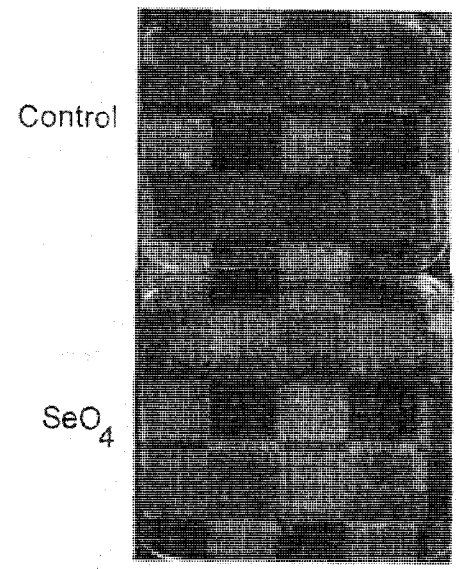
Edited by C. Guy

Figure S1. Arabidopsis (ecotype WS) growth on media plates without (control) and with selenate after 10 days.



Please note that the size and format of Table S1 prevented the conversion of the excel file to a viewable pdf file. Table S1 can be viewed on the publisher's website:

http://www.blackwell-synergy.com/doi.abs/10.1111/j1399-3054.2007.01002x

Table S2. List of Gene Ontology (GO) Biological Processes terms were tested for over-representation using the hypergeometric test. Categories were created using the list of differentially expressed genes identified in root (A) and shoot (B) tissue. The Odds Ratio is calculated as follows: (# of Se-responsive genes in GO term category/# of all Se-responsive genes in the genome) divided by (# of non-Se-responsive genes in GO term category/# of all non-Se-responsive genes in the genome). An odds ratio greater than 1 means that the GO term category is over-represented among the genes differentially expressed by Se relative to the non-differentially expressed genes.

A.

ROOTS

ID	Term	Pvalue	Odds Ratio	Count: Se-responsive	Count: Genome
GO:0006950	response to stress	4.16E-17	2.75	108	1077
GO:0050896	response to stimulus	1.50E-16	2.26	159	1941
GO:0009628	response to abiotic stimulus	1.06E-15	2.47	120	1320
GO:0042221	response to chemical stimulus	3.90E-13	2.60	87	895
GO:0009266	response to temperature stimulus	1.41E-11	4.54	34	208
GO:0006952	defense response	2.05E-11	3.03	56	492
GO:0006800	reactive oxygen species metabolism	8.68E-11	4.90	29	166
GO:0006979	response to oxidative stress	2.63E-10	4.99	27	152
GO:0009607	response to biotic stimulus	3.78E-10	2.62	62	620
GO:0009408	response to heat	1.51E-09	7.94	17	66
GO:0009861	jasmonic acid and ethylene-dependent pathways	6.47E-09	5.16	22	120
GO:0009611	response to wounding	1.03E-08	4.25	26	167
GO:0009814	defense response to pathogen	7.00E-08	3.81	26	183
GO:0009723	response to ethylene stimulus	9.14E-08	4.78	20	116
GO:0006970	response to osmotic stress	1.25E-07	3.81	25	176
GO:0009605	response to external stimulus	1.59E-07	3.26	30	242
GO:0042829	defense response to pathogen	2.27E-07	2.84	36	329
GO:0009613	response to pest, pathogen or parasite	5.58E-07	2.51	42	431
GO:0009753	response to jasmonic acid stimulus	5.84E-07	4.63	18	107
GO:0009737	response to abscisic acid stimulus	8.58E-07	3.61	23	169
GO:0009651	response to salt stress	9.99E-07	3.71	22	158
GO:0042828	response to pathogen	1.09E-06	2.53	39	396
GO:0051707	response to other organism	1.43E-06	2.33	45	494
GO:0045449	regulation of transcription	3.26E-06	1.74	92	1339
GO:0000103	sulfate assimilation	4.37E-06	22.62	6	12
GO:0019219	regulation of nucleic acid metabolism	4.56E-06	1.73	92	1350
GO:0019222	regulation of metabolism	6.56E-06	1.70	94	1400
GO:0031323	regulation of cellular metabolism	7.80E-06	1.70	93	1387
GO:0006791	sulfur utilization	7.82E-06	19.39	6	13
GO:0051244	regulation of cellular physiological process	1.33E-05	1.65	99	1521
GO:0009725	response to hormone stimulus	1.59E-05	2.16	42	492
GO:0050794	regulation of cellular process	1.65E-05	1.64	99	1529
GO:0050791	regulation of physiological process	2.18E-05	1.62	100	1559
GO:0006350	transcription	2.34E-05	1.65	92	1407
GO:0009719	response to endogenous stimulus	3.48E-05	1.91	53	698
GO:0009409	response to cold	3.82E-05	3.29	18	143
GO:0006355	regulation of transcription, DNA-dependent	4.99E-05	1.83	57	779
GO:0009873	ethylene mediated signaling pathway	8.23E-05	5.83	9	44
GO:0010035	response to inorganic substance	9.52E-05	4.19	12	77
GO:0006351	transcription, DNA-dependent	1.21E-04	1.77	57	805
GO:0050789	regulation of biological process	1.26E-04	1.53	102	1671
GO:0000160	two-component signal transduction system	2.70E-04	4.36	10	62
GO:0009694	jasmonic acid metabolism	2.92E-04	11.29	5	15
GO:0009695	jasmonic acid biosynthesis	2.92E-04	11.29	5	15
GO:0031407	oxylipin metabolism	2.92E-04	11.29	5	15
GO:0031408	oxylipin biosynthesis	2.92E-04	11.29	5	15

GO:0009765	photosynthesis, light harvesting	3.19E-04	7.98	6	23
GO:0009751	response to salicylic acid stimulus	3.22E-04	3.40	13	100
GO:0009646	response to absence of light	7.24E-04	33.81	3	5
GO:0046686	response to cadmium ion	7.57E-04	4.64	8	47
GO:0009414	response to water deprivation	7.74E-04	3.24	12	96
GO:0015979	photosynthesis	8.50E-04	3.20	12	97
GO:0009415	response to water	1.57E-03	2.96	12	104
GO:0010038	response to metal ion	1.86E-03	3.58	9	66
GO:0009612	response to mechanical stimulus	2.38E-03	16.90	3	7
GO:0016567	protein ubiquitination	3.42E-03	3.23	9	72
GO:0006972	hyperosmotic response	3.63E-03	5.64	5	25
GO:0010200	response to chitin	3.68E-03	13.52	3	8
GO:0009739	response to gibberellic acid stimulus	3.87E-03	2.94	10	87
GO:0031347	regulation of defense response	5.29E-03	45.02	2	3
GO:0009664	cell wall organization and biogenesis	5.77E-03	2.76	10	92
GO:0048527	lateral root development	6.22E-03	6.44	4	18
GO:0048528	post-embryonic root development	7.62E-03	6.01	4	19
GO:0009893	positive regulation of metabolism	8.17E-03	4.51	5	30
GO:0007047	cell wall organization and biogenesis	8.89E-03	2.10	15	177
GO:0045229	external structure organization and biogenesis	8.89E-03	2.10	15	177
GO:0005992	trehalose biosynthesis	9.20E-03	5.64	4	20
GO:0043119	positive regulation of physiological process	9.40E-03	3.23	7	56

B.

SHOOTS				Count: Se-responsive	Count: Genome
ID	Term	Pvalue	OddsRatio		
GO:0042221	response to chemical stimulus	7.89E-11	3.33	46	895
GO:0050896	response to stimulus	8.29E-10	2.46	72	1941
GO:0009628	response to abiotic stimulus	2.34E-09	2.70	55	1320
GO:0006810	transport	5.84E-08	2.27	65	1853
GO:0051179	localization	7.43E-08	2.25	65	1865
GO:0051234	establishment of localization	7.43E-08	2.25	65	1865
GO:0009719	response to endogenous stimulus	3.23E-07	2.95	33	698
GO:0009725	response to hormone stimulus	7.94E-07	3.27	26	492
GO:0006950	response to stress	3.29E-06	2.36	41	1077
GO:0009636	response to toxin	3.99E-06	10.06	8	53
GO:0006118	electron transport	5.13E-06	3.08	24	478
GO:0009737	response to abscisic acid stimulus	1.05E-05	4.76	13	169
GO:0019748	secondary metabolism	2.35E-05	3.50	17	296
GO:0006865	amino acid transport	5.08E-05	8.23	7	55
GO:0015837	amine transport	5.08E-05	8.23	7	55
GO:0006091	generation of precursor metabolites and energy	5.51E-05	2.39	29	737
GO:0009404	toxin metabolism	7.95E-05	9.65	6	41
GO:0009407	toxin catabolism	7.95E-05	9.65	6	41
GO:0015849	organic acid transport	8.03E-05	7.59	7	59
GO:0046942	carboxylic acid transport	8.03E-05	7.59	7	59
GO:0007582	physiological process	1.16E-04	1.53	203	9577
GO:0009914	hormone transport	2.32E-04	10.39	5	32
GO:0009926	auxin polar transport	2.32E-04	10.39	5	32
GO:0030026	manganese ion homeostasis	3.14E-04	Inf	2	2
GO:0006970	response to osmotic stress	3.16E-04	3.78	11	176
GO:0009605	response to external stimulus	4.06E-04	3.23	13	242
GO:0051707	response to other organism	5.60E-04	2.42	20	494
GO:0042398	amino acid derivative biosynthesis	6.91E-04	4.04	9	135
GO:0009699	phenylpropanoid biosynthesis	7.31E-04	5.12	7	84
GO:0045333	cellular respiration	7.57E-04	7.79	5	41
GO:0031667	response to nutrient levels	7.81E-04	11.20	4	24
GO:0019725	cell homeostasis	7.85E-04	5.05	7	85
GO:0006972	hyperosmotic response	9.17E-04	10.66	4	25
GO:0009744	response to sucrose stimulus	9.17E-04	10.66	4	25
GO:0006882	zinc ion homeostasis	9.31E-04	111.38	2	3
GO:0009814	defense response to pathogen	1.63E-03	3.27	10	183
GO:0009755	hormone-mediated signaling	1.75E-03	3.51	9	154
GO:0009991	response to extracellular stimulus	1.85E-03	8.61	4	30
GO:0042592	homeostasis	1.92E-03	4.28	7	99
GO:0042828	response to pathogen	2.00E-03	2.40	16	396
GO:0006519	amino acid and derivative metabolism	2.05E-03	2.39	16	397
GO:0045454	cell redox homeostasis	2.10E-03	8.29	4	31
GO:0009735	response to cytokinin stimulus	2.24E-03	5.96	5	52
GO:0019438	aromatic compound biosynthesis	2.25E-03	3.70	8	130
GO:0009267	cellular response to starvation	2.61E-03	12.88	3	16
GO:0031669	cellular response to nutrient levels	2.61E-03	12.88	3	16
GO:0009753	response to jasmonic acid stimulus	2.98E-03	3.94	7	107
GO:0006772	thiamin metabolism	3.03E-03	37.12	2	5
GO:0009228	thiamin biosynthesis	3.03E-03	37.12	2	5
GO:0042723	thiamin and derivative metabolism	3.03E-03	37.12	2	5
GO:0042724	thiamin and derivative biosynthesis	3.03E-03	37.12	2	5
GO:0009698	phenylpropanoid metabolism	3.31E-03	3.86	7	109
GO:0009607	response to biotic stimulus	3.68E-03	2.01	21	620
GO:0005975	carbohydrate metabolism	3.87E-03	1.96	22	664
GO:0009738	abscisic acid mediated signaling	4.05E-03	6.78	4	37
GO:0006857	oligopeptide transport	4.20E-03	5.09	5	60
GO:0031668	cellular response to extracellular stimulus	4.35E-03	10.47	3	19

GO:0051716	cellular response to stimulus	4.35E-03	10.47	3	19
GO:0006575	amino acid derivative metabolism	4.44E-03	3.02	9	177
GO:0015833	peptide transport	4.51E-03	5.00	5	61
GO:0009613	response to pest, pathogen or parasite	4.57E-03	2.19	16	431
GO:0009987	cellular process	4.76E-03	1.35	182	8927
GO:0009861	jasmonic acid systemic resistance	5.60E-03	3.48	7	120
GO:0042594	response to starvation	5.81E-03	9.30	3	21
GO:0050875	cellular physiological process	5.90E-03	1.34	176	8626
GO:0042829	defense response to pathogen	6.14E-03	2.33	13	329
GO:0001666	response to hypoxia	6.22E-03	22.27	2	7
GO:0009414	response to water deprivation	7.27E-03	3.74	6	96
GO:0009651	response to salt stress	7.31E-03	3.00	8	158
GO:0042538	hyperosmotic salinity response	7.54E-03	8.37	3	23
GO:0007154	cell communication	8.04E-03	1.81	23	751
GO:0009809	lignin biosynthesis	8.50E-03	7.97	3	24
GO:0009624	response to nematode	9.53E-03	5.20	4	47

Table S3. Comparison of microarry and macroarray analysis for the 31 selected selenate-responsive genes. For both gene-expression experiments, Arabidopsis plants were grown on control media and selenate. Root and shoot tissue were separated and analyzed. For this calculation, fold-change of down-regulated transcripts were converted into a negative value, i.e. 0.5 became -2. Shown is the mean fold change for two replicate experiments for each analysis. Data were subjected to regression analysis (R2 = 0.52, p-value <0.0001).

Root tissue			
locus	**annotation**	**microarray**	**macroarray**
At1g56600	water stress related protein	18.80	2.43
At5g43840	HSP TF	10.64	2.24
At1g05680	auxin signaling	4.39	2.76
At1g02850	glycosyl transferase	6.61	2.96
At4g11650	osmotin	3.30	1.91
At2g29490	GSH transferase	3.09	2.13
At5g12030	HSP 17.6	2.88	2.12
At5g52300	low temp induced protein	5.69	3.99
At5g13170	senescence protein	3.37	2.99
At1g23730	carbonic anhydrase	3.01	4.07
At3g51860	Ca/H antiporter	5.11	4.09
At3g63440	cytokinin oxidase	0.11	0.31
At5g44120	legumin-like protein	0.03	0.16
At5g38710	proline oxidase	0.34	0.75

Shoot tissue			
locus	**annotation**	**microarray**	**macroarray**
At4g34410	ERB (ethylene receptor) TF	263.44	2.41
At1g12610	Cold binding factor 1 TF	227.79	2.19
At5g12020	HSP 17.6	43.21	2.00
At4g33720	PR protein	47.14	24.29
At2g26150	HSP TF	21.58	2.62
At3g46230	HSP17	16.67	2.86
At1g59860	HSP17	10.10	2.05
At1g53540	HSP 17.6	7.61	2.16
At3g12580	HSP70	3.96	4.62
At4g11280	ACC synthase	16.54	2.23
At1g27730	salt tolerant protein	29.25	4.10
At4g02200	drought induced protein	3.14	2.40
At5g51440	mitochondria HSP	3.04	3.68
At3g08940	Chl a/b binding protein	0.16	0.61
At4g02770	PSI D protein	0.31	0.30
At3g01500	chloroplast carbonic anhydrase	0.03	0.63
At1g52820	giberellin oxidase	0.06	0.30
At5g04960	pectinesterase	0.28	0.22
At2g46690	auxin regulated protein	0.34	0.53

Foreword to Chapter 5

As evinced in Chapter 4, a microarray analysis revealed that nearly 900 and 400 genes are differentially regulated in root and shoot tissue respectively when Arabidopsis plants are treated with selenate. Interestingly, CpNifS was not identified as one of these genes! One explanation is that CpNifS is regulated post-transcriptionally, i.e. at the protein level. However, I can rule this out since CpNifS protein levels did not increase when plants were grown on selenate (unpublished results). Thus, it may be likely that although CpNifS overexpression can increase selenium tolerance, the true function of this protein at endogenous levels in Arabidopsis may not confer protection again selenium. This prompted me to reconsider the real function of CpNifS and investigate if the protein has a role in chloroplastic FeS protein maturation. This is the subject of Chapter 5.

Chloroplast iron-sulfur cluster protein maturation requires the essential cysteine desulfurase CpNifS

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NifS-like proteins provide the sulfur (S) for the formation of iron-sulfur (Fe-S) clusters, an ancient and essential type of cofactor found in all three domains of life. Plants are known to contain two distinct NifS-like proteins, localized in the mitochondria (MtNifS) and the chloroplast (CpNifS). In the chloroplast, five different Fe-S cluster types are required in various proteins. These plastid Fe-S proteins are involved in a variety of biochemical pathways including photosynthetic electron transport and nitrogen and sulfur assimilation. *In vitro*, the chloroplastic cysteine desulfurase CpNifS can release elemental sulfur from cysteine for Fe-S cluster biogenesis in ferredoxin. However, because of the lack of a suitable mutant allele, the role of CpNifS has not been studied thus far *in planta*. To study the role of CpNifS in Fe-S cluster biogenesis *in vivo*, the gene was silenced by using an inducible RNAi (interference) approach. Plants with reduced CpNifS expression exhibited chlorosis, a disorganized chloroplast structure, and stunted growth and eventually became necrotic and died before seed set. Photosynthetic electron transport and carbon dioxide assimilation were severely impaired in the silenced plant lines. The silencing of CpNifS decreased the abundance of all chloroplastic Fe-S proteins tested, representing all five Fe-S cluster types. Mitochondrial Fe-S proteins and respiration were not affected, suggesting that mitochondrial and chloroplastic Fe-S assembly operate independently. These findings indicate that CpNifS is necessary for the maturation of all plastidic Fe-S proteins and, thus, essential for plant growth.

Fe-S proteins | inducible RNAi | photosynthesis | *Arabidopsis thaliana*

NifS-like proteins have cysteine desulfurase activity, which releases elemental sulfur (S) from the amino acid cysteine for the formation of iron-sulfur (Fe-S) clusters. First discovered in the nitrogen-fixing bacterium *Azotobacter vinelandii* (1), NifS-like proteins now represent a conserved group of proteins that are found in all of the domains of life (2). In bacteria, NifS-like proteins can be broadly classified on the basis of primary structure as either group I, which have a proposed general housekeeping role, or group II, which are likely required during oxidative stress. *Escherichia coli* contains 3 NifS-like proteins: IscS (group I), CsdA (group II), and SufS (group II) (for reviews see refs. 2 and 3). It is noteworthy that deletion of IscS is not lethal in *E. coli*; this mild phenotype is attributed to complementation by SufS (4).

In plants, Fe-S proteins are known to exist in the mitochondria, cytosol, and chloroplasts. The chloroplast contains five Fe-S cluster types: ferredoxin (Fd)-type 2Fe-2S, Rieske-type 2Fe-2S, 3Fe-4S, 4Fe-4S, and the siroheme 4Fe-4S (5, 6). These Fe-S clusters are used by an assortment of chloroplastic proteins that are involved in a diverse range of functions including protein import, sulfur and nitrogen reduction, chlorophyll synthesis, and photosynthetic electron transport (5).

Mitochondria and chloroplasts are thought to be the result of separate endosymbiotic events during the evolution of eukaryotes. Plants contain two distinct NifS-like proteins, one localized to mitochondria (MtNifS) (7) and the other localized to chloroplasts (CpNifS) (8, 9). MtNifS (group I) is most similar to bacterial IscS. The MtNifS homologue in yeast is required for Fe-S cluster formation in the mitochondria and cytosol, and MtNifS may function similarly in plants (7). CpNifS is a group II NifS-like protein (9). *In vitro*, CpNifS can provide the S for Fe-S cluster insertion into apo-Fd to form functional holo-Fd (10). Similar to the bacterial SufS, CpNifS cysteine desulfurase specific activity is low but greatly stimulated by a SufE protein (11, 12).

It may be hypothesized that CpNifS provides the sulfur for all five types of Fe-S proteins that occur in plastids. However, the specific function of CpNifS in chloroplastic Fe-S cluster assembly and its significance for plant development and survival has not been established *in planta* because of the lack of a suitable mutant. To determine the role of CpNifS in the synthesis of chloroplastic Fe-S clusters, we silenced the *Arabidopsis CpNifS* gene by using an ethanol-inducible RNAi construct and investigated the effects on levels of Fe-S proteins and photosynthesis.

Results

CpNifS Is Essential for Plant Growth and Maintenance of Chloroplast Structure. Initially, *CpNifS* was silenced constitutively by RNAi using the cauliflower mosaic virus *35S* promoter. Lines in which CpNifS expression was significantly reduced displayed severely chlorotic cotyledons and died as seedlings. Although plants with milder phenotypes could be propagated, we found these constitutively driven *CpNifS* RNAi lines to be unstable for the trait. To avoid these problems, and to be able to study the effects of complete *CpNifS* silencing at a later developmental stage, an ethanol-inducible RNAi construct was used to silence *CpNifS* in *Arabidopsis* plants. Eleven transgenic inducible *CpNifS* RNAi lines were obtained and bred to homozygosity. Two of these lines, *CpNifS-6* and *CpNifS-9*, were selected for further studies. To determine the efficacy of the inducible construct, wild-type (WT) and the two selected transgenic RNAi lines were grown for 2 weeks and then induced by ethanol treatment; control plants were not treated with ethanol. WT and transgenic plants that were not induced with ethanol typically did not show any signs of stress (Fig. 1*A*) and had normal development and seed production. Indeed, cytosolic ascorbate peroxidase I, a marker of oxidative stress in plants (13), was not induced in ethanol-

Author contributions: M.P. and E.A.H.P.-S. contributed equally to this work; D.V.H., M.P., and E.A.H.P.-S. designed research; D.V.H., S.E.A.-G., C.M.C., S.K.H., and P.K. performed research; S.K.H and P.K. contributed new reagents/analytic tools; D.V.H. and C.M.C. analyzed data; and D.V.H., M.P., and E.A.H.P.-S. wrote the paper.

The authors declare no conflict of interest.

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Abbreviations: Fd, ferredoxin; MtNifS, mitochondrial NifS-like protein; CpNifS, chloroplastic NifS-like protein; PSI/II, photosystem I/II; ETR, electron-transport rate; RRF, ribosome-recycling factor; Rubisco, ribulose bisphosphate carboxylase oxygenase; SiR, sulfite reductase; NiR, nitrite reductase; GOGAT, 2-oxoglutarate aminotransferase.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0700774104/DC1.

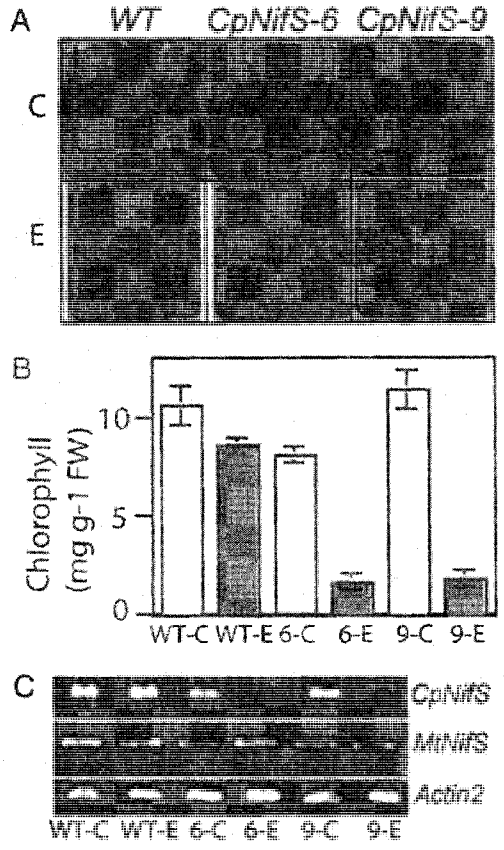

Fig. 1. Phenotypes of *CpNifS*-silenced plants. (*A*) Two-week old WT, *CpNifS-6*, and *CpNifS-9* plants were treated with 2% ethanol (E) every 4 days for 3 weeks. Control plants (C) were treated with water. (*B*) Chlorophyll content in plants treated with ethanol or control plants. $P < 0.05$ for ethanol-treated *CpNifS-6* and *CpNifS-9*. (*C*) *CpNifS*, *MtNifS*, and *Actin2* transcript detection by RT-PCR 1 week after the start of ethanol treatment. FW, fresh weight.

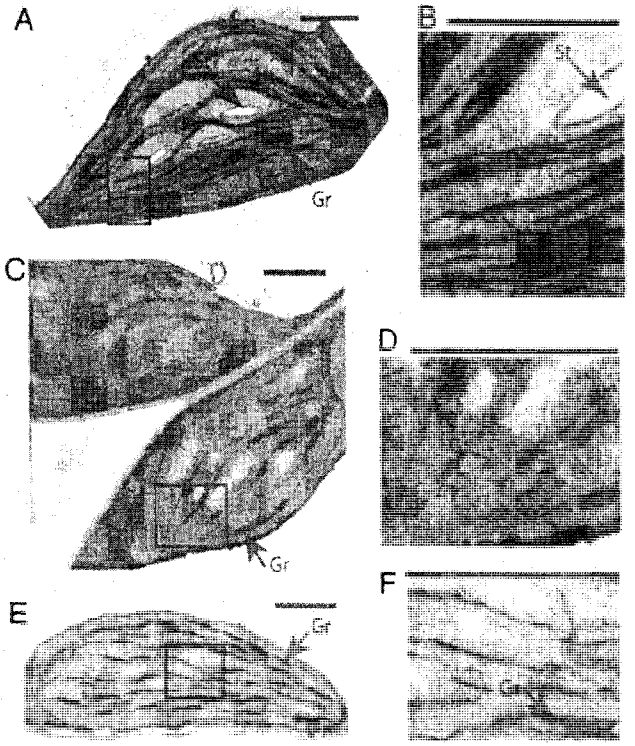

Fig. 2. Chloroplast ultrastructure is altered in *CpNifS*-silenced plants. Leaf samples were fixed 10 days after ethanol treatment of WT (*A* and *B*), *CpNifS-6* (*C* and *D*), and *CpNifS-9* (*E* and *F*) plants. Thin sections were examined by transmission electron microscopy. *B*, *D*, and *F* show magnifications of the boxed areas in *A*, *C*, and *E*, respectively. St, starch granules; Gr, grana. (Scale bars, 1 μm.)

treated plants [see supporting information (SI) Fig. 6]. Only in rare cases was slight chlorosis observed in uninduced transgenics, possibly because of leakiness of the ethanol-inducible promoter. After 3 weeks of ethanol treatment, the *CpNifS-6* and *CpNifS-9* transgenics showed severely stunted growth, chlorosis, and leaf necrosis. Ethanol-treated WT plants showed no such symptoms, albeit that ethanol-treated WT plants were sometimes slightly smaller after 3 weeks of treatment (Fig. 1*A*). At this stage, the leaves of ethanol-treated *CpNifS-6* and *CpNifS-9* plants had at least 5-fold lower chlorophyll content compared with WT and untreated control plants (Fig. 1*B*). When ethanol treatment was stopped at this stage, the *CpNifS* plants recovered and were able to set seed. However, continued ethanol treatment ultimately resulted in irreversible damage and death of the RNAi transgenics, whereas it had no visible or only a marginal effect on the WT.

To determine whether the stunted growth of ethanol-treated transgenic plants coincided with the loss of *CpNifS* mRNA, leaf samples were taken from treated and untreated plants at week 3 of plant growth (1 week after the start of ethanol treatment) for RT-PCR analysis. The *CpNifS* transcript was not detected in leaves of chlorotic *CpNifS-6* and *CpNifS-9* plants that were treated with ethanol (Fig. 1*C*). The mRNAs for Actin2 and MtNifS (NFS1) were not affected by ethanol treatment, showing that the RNAi ethanol-inducible construct is specific for the *CpNifS* gene product.

The severe chlorosis displayed by the ethanol-induced *CpNifS*-silenced plants prompted us to analyze their ultrastructure by transmission electron microscopy to determine the effects of CpNifS deficiency on leaf cell and plastid structure. The overall cell shape and the morphology of the nucleus appeared the same in ethanol-treated WT, *CpNifS-6*, and *CpNifS-9* plants; it is also noteworthy that the structure of the mitochondria was unchanged in *CpNifS* plants, suggesting that MtNifS and mitochondrial processes were not affected (data not shown). However, chloroplast structure was drastically changed in plants in which *CpNifS* was silenced (Fig. 2). Compared with the discrete and stacked thylakoid membrane grana displayed by WT, the grana in silenced *CpNifS-6* and *CpNifS-9* plants were hypertrophied and dissociated from each other. Also of interest was the absence of starch granules in *CpNifS-6* and *CpNifS-9*, likely caused by a disruption of photosynthesis.

Taken together, these results strongly suggest that CpNifS is an essential protein in *Arabidopsis*. CpNifS loss of function causes pleiotropic phenotypes and eventually plant death. To analyze the primary cause of these phenotypes and to insight into the direct function of CpNifS in plants before pleiotropic phenotypes were apparent, further experiments with the inducible RNAi lines were performed by using plants in which ethanol treatment started 3 weeks after germination, analyzing the plants 10 days after induction.

CpNifS Mutants Have Impaired Photosynthesis but Unaltered Respiration. We hypothesized that *CpNifS* silencing would affect photosynthesis, because photosynthetic electron transport requires many Fe-S proteins, particularly in photosystem I (PSI). To determine how loss of CpNifS affects photosynthetic electron

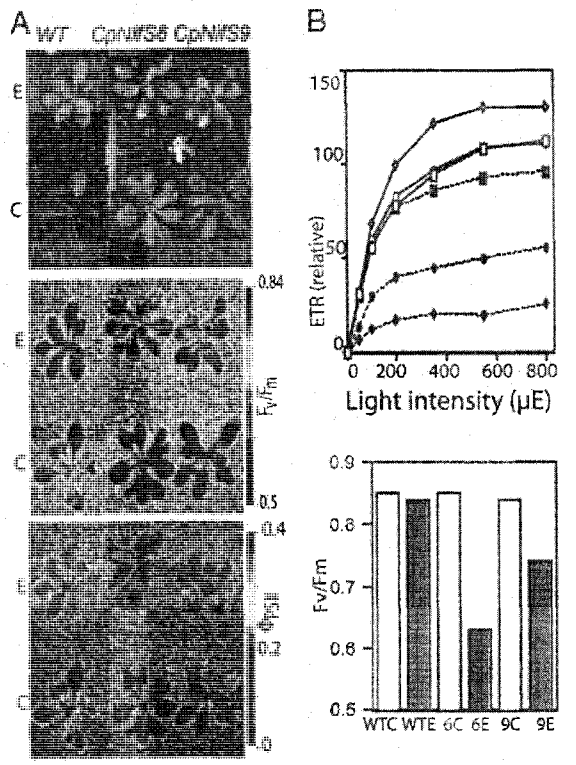

Fig. 3. Chlorophyll fluorescence analysis. (*A*) Chlorophyll fluorescence imaging. Shown are a regular-color photograph (*Top*) and false-color images for Fv/Fm (*Middle*) and Φ_{PSII} (*Bottom*) of the same plants. False-color scales for fluorescence parameters are shown to the right (red color represents the highest values and blue represents the lowest values). (*B Upper*) Relative ETR at varying light intensities. Squares, diamonds, and circles represent WT, *CpNifS-6*, and *CpNifS-9*, respectively. Open symbols correspond to untreated controls, and closed symbols signify ethanol treatment. (*B Lower*) Fv/Fm. Note: standard errors were too small to be plotted. $P < 0.05$ for ethanol-treated *CpNifS-6* and *CpNifS-9*. C, control plants; E, ethanol-treated plants.

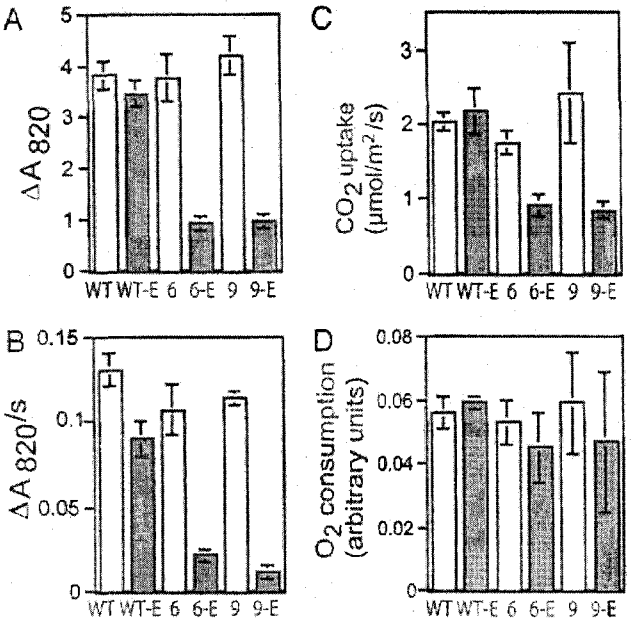

Fig. 4. PSI activity and CO_2 assimilation. WT, 6, and 9 indicate control plants for WT, *CpNifS-6*, and *CpNifS-9* respectively, and WT-E, 6-E, and 9-E indicate the respective ethanol-treated plants. (*A*) PSI activity. Shown is the extent of the ΔA_{820} (absorbance change at 820 nm, indicative of the P700 activity of PSI) induced with a flash of saturating light, in relative units. (*B*) Rate of reduction of PSI by upstream electron donors. Values, in relative units, represent the initial rate of the absorbance change at 820 nm ($\Delta A_{820}/s$) in darkness after complete photooxidation of P700, which results mainly from reduction of $P700^+$ by upstream electron donors. (*C*) Leaf photosynthetic CO_2 uptake. (*D*) Leaf respiratory O_2 consumption. $P < 0.05$ for ethanol-treated 6-E and 9-E for *A*–*C*. All values are the average and standard error of five independent measurements.

transport in intact plants, a chlorophyll fluorescence imaging system was used. For silenced plants and controls, images were captured of two chlorophyll fluorescence parameters: Fv/Fm, which indicates the maximum photochemical efficiency (intactness) of PSII, and Φ_{PSII}, which indicates the fraction of PSII complexes available for photochemistry and indirectly measures the flux of electrons out of PSII (14).

Transgenic plants that were ethanol-treated showed chlorosis, particularly in younger leaves (Fig. 3*A*). However, the older leaves that had already fully expanded before induction had not become chlorotic at this stage (10 days after induction). Accordingly, when treated with ethanol, Fv/Fm was strongly reduced in the younger leaves of *CpNifS-6* and *CpNifS-9* transgenics compared with WT and untreated controls, but older leaves were less affected (Fig. 3*A*). The electron flow, Φ_{PSII}, was reduced in both the young and the older leaves of the transgenics, compared with WT.

To gain more quantitative insight into how *CpNifS* silencing affects photosynthesis, Φ_{PSII} was measured and used to estimate the electron-transport rate (ETR) over a range of light intensities in expanded leaves in which chlorosis was absent or minimal. At all light intensities the Φ_{PSII} and, as a consequence, ETR were reduced; ETR saturated at much lower light intensities in *CpNifS-6* and *CpNifS-9* plants induced with ethanol, compared with WT and untreated plants (Fig. 3*B*). The reduced ETR correlated with a deficiency in *CpNifS* mRNA in the same leaves (Fig. 1*C*). The *CpNifS*-silenced plants also showed a decrease in Fv/Fm (Fig. 3*C*), suggesting photoinhibition or some other effect that reduced the maximum photochemical efficiency of PSII.

All of the Fe-S proteins involved in photosynthetic electron transport are found downstream of PSII: one 2Fe-2S cluster is in the Rieske protein of the cytochrome b_6f complex, and three 4Fe-4S clusters are found in PSI (5). The photochemical activity of PSI was analyzed in leaf disks by measuring the light-induced absorbance change at 820 nm (ΔA_{820}), which occurs with photooxidation of the P700 reaction center of PSI (15, 16). Ethanol-treated *CpNifS-6* and *CpNifS-9* plants had less than one third of the ΔA_{820} exhibited by ethanol-treated WT or untreated plants (Fig. 4*A*), which indicates a substantial loss of photochemically active P700. The rate of dark reduction of P700 after the oxidizing flash was also reduced in the *CpNifS*-silenced plants, indicating that the flow of electrons into PSI from upstream donors was slower (Fig. 4*B*). To test whether the reduced electron-transport activity limited plant productivity, we measured photosynthetic CO_2 fixation. Indeed, the CO_2 fixation rate in the light was reduced by 50% in the ethanol-treated *CpNifS-6* and *CpNifS-9* plants compared with WT and untreated plants (Fig. 4*C*). However, oxygen consumption in the dark, which is indicative of mitochondrial respiration, was not affected (Fig. 4*D*). Thus, *CpNifS* silencing does not appear to disrupt mitochondrial function, while severely affecting chloroplast function.

Levels of Chloroplastic Fe-S Proteins Are Reduced by *CpNifS* Silencing. We next investigated the direct effect of *CpNifS* silencing on the abundance of chloroplast proteins with or without Fe-S clusters (Fig. 5*A*). Consistent with an absence of *CpNifS* mRNA, very little CpNifS protein was detected in transgenic plants treated

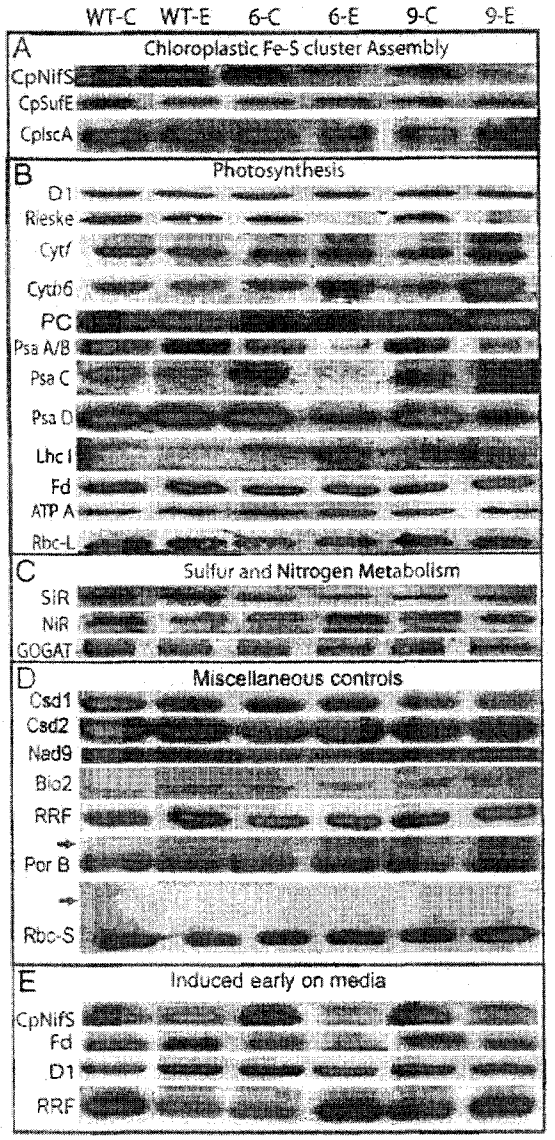

Fig. 5. Effect of *CpNifS* silencing on protein levels. Immunoblot analysis for selected proteins is shown. Lanes labeled WT-C, 6-C, and 9-C indicate control plants for WT, *CpNifS-6*, and *CpNifS-9* respectively, and lanes labeled WT-E, 6-E, and 9-E indicate the respective ethanol-treated plants. (*A–D*) Samples from plants grown on soil either under control conditions or after treatment with 2% ethanol for 10 days: (*A*) Chloroplastic Fe-S cluster assembly; (*B*) photosynthesis; (*C*) sulfur and nitrogen metabolism; (*D*) miscellaneous controls. (*E*) Protein extracts from plants grown from germination on 0.5 Murashige and Skoog medium + 1% sucrose plates with or without ethanol. Total protein extracts (25 μg of protein) were separated by SDS/PAGE and blotted to nitrocellulose before immunostaining with the indicated antibodies. RRF was used as a loading control. D1, D1 subunit of PSII; PC, plastocyanin; ATP A, ATP synthase subunit A; Lhcl, light-harvesting complex proteins of PSI; Rbc-L, large subunit of Rubisco; Csd, Cu/Zn superoxide dismutase; Nad9, subunit 9 of mitochondrial complex 1; Bio2, mitochondrial biotin synthase; PsaA–D, PSI subunits A–D; Por B, protochlorophyllide oxidoreductase; Rbc-S, small subunit of Rubisco. Specific bands were identified by immunostaining and by their molecular weight. Arrows indicate where precursor proteins would have been predicted to accumulate. For each protein, the relevant section of one representative blot of three independent repeats is shown.

with ethanol. The abundance of the CpNifS activator, CpSufE, was decreased in the absence of CpNifS, compared with WT and untreated controls. In contrast, the relative abundance of CpIscA, a putative scaffold protein for chloroplastic Fe-S cluster formation (17), was hardly affected by *CpNifS* silencing.

To identify which specific steps of photosynthesis may be affected by the silencing of *CpNifS*, we examined the abundance of a variety of proteins involved in photosynthesis (Fig. 5*B*). *CpNifS* silencing did not affect the D1 protein, a component of the PSII reaction center in which no Fe-S clusters are present (18), suggesting that most PSII was intact. In contrast, components of the cytochrome b_6f complex were affected by *CpNifS* silencing. Most noteworthy is the near absence of the 2Fe-2S Rieske protein. Although the abundance of cytochrome *f* was not affected, cytochrome b_6 was more abundant in *CpNifS*-silenced plants. Both cytochrome *f* and cytochrome b_6 are heme (Fe) proteins and chloroplast-encoded. A very profound effect of *CpNifS* silencing was observed in PSI. PsaA, PsaB, and PsaC, all 4Fe-4S proteins, were strongly reduced or absent in CpNifS-silenced plants. The PsaD protein, which is not an Fe-S cluster protein but thought to be involved in Fd docking (19), was reduced also. This reduction in PsaD abundance may reflect the overall lack of integrity of PSI in *CpNifS*-silenced plants. Perhaps to compensate for the very low abundance of PSI reaction-center proteins in *CpNifS*-silenced plants, there was an increased abundance of subunits of the light-harvesting complex proteins of PSI (LhcI).

Surprisingly, no effect was seen on the abundance of the 2Fe-2S protein Fd in soil-grown *CpNifS*-silenced plants. One possible explanation is that the Fd already present in the 3-week-old plants before ethanol induction was very stable, so that Fd levels did not go down once further production of holo-Fd ceased. To test this possibility, we induced *CpNifS* silencing during germination on media plates. After 1 week, ethanol-treated transgenic plants had extreme chlorosis and stunted growth compared with WT and untreated plants. Western blotting showed that CpNifS and Fd were absent in these ethanol-treated transgenics, indicating that Fd levels do decrease if *CpNifS* is silenced at an early stage in plant development (Fig. 5*E*). Therefore, holo-Fd may be very stable and remain abundant even when the production of its cofactor is impeded. D1, a plastid- encoded subunit of PSII, and ribosome-recycling factor (RRF), which is a nuclear-encoded plastid protein, were not affected by *CpNifS* silencing from germination.

In the soil-grown plants that were ethanol-induced at the 3-week-old stage, the ATP-A subunit of chloroplastic ATP synthase and the large subunit of ribulose bisphosphate carboxylase oxygenase (Rubisco), involved in photosynthetic ATP production and CO_2 fixation, respectively, were not affected; neither of these are Fe-S proteins (Fig. 5*B*). There was also no major effect on plastocyanin, the copper protein that is active in electron transport between cytochrome b_6f and PSI (Fig. 5*B*). Two other copper-containing proteins, Cu/ZnSOD1 and Cu/ZnSOD2, involved in free radical scavenging, were also not affected by *CpNifS* silencing (Fig. 5*D*).

In addition to the photosynthetic proteins described above, other plastidic Fe-S cluster proteins are expected to depend on CpNifS for their maturation. Indeed, the 3Fe-4S cluster protein Fd-glutamine-2-oxoglutarate aminotransferase (GOGAT) was slightly less abundant compared with WT and untreated controls. Moreover, sulfite reductase (SiR) and, to a lesser extent, nitrite reductase (NiR) were reduced in *CpNifS*-silenced plants (Fig. 5*C*). SiR and NiR contain the siroheme 4Fe-4S cluster type unique to plants. The polypeptide level of NiR was reduced only moderately (30%) compared with WT and untreated plants. In contrast, the enzyme activity of NiR was significantly reduced, by 70% in *CpNifS-6* and 45% in *CpNifS-9*, compared with untreated transgenic plants (SI Fig. 7). The observation that the activity of NiR was reduced to a greater extent than NiR protein levels suggests that apo-NiR, lacking the Fe-S cofactor, had accumulated in CpNifS-deficient plants.

The levels of CpNifS and several Fe-S proteins were monitored over a 10-day period after ethanol induction to determine how quickly CpNifS decreased in *CpNifS-6* plants and how quickly it was followed by a decrease in chloroplastic Fe-S proteins. CpNifS began to diminish in transgenic plants 1 day after ethanol induction and had almost completely disappeared by day 10 (SI Fig. 8). Protein abundance of PSI subunits PsaA/B began to decrease on day 2 after ethanol induction, whereas decline of PsaC levels started on day 7. By day 10, a slight reduction in GOGAT, NiR, and SiR was seen. Levels of the control protein RRF remained constant over the course of 10 days.

To determine whether the decrease in chloroplastic Fe-S proteins was caused by a decrease in mRNA, a Northern blot was performed to detect the transcript abundance of Rieske, GOGAT, and SiR. A decrease in mRNA was not observed (SI Fig. 6) despite a large reduction in Rieske, GOGAT, and SiR protein levels (Fig. 5 *B* and *C*), which suggests that these Fe-S proteins need their appropriate cofactor to be stable and supports the hypothesis that CpNifS is critical for Fe-S cluster formation in the chloroplast.

In view of the decrease in several abundant Fe-S proteins in the *CpNifS*-silenced lines, leaf nutrient status was investigated (SI Table 1). We did not see a reduction in total leaf iron content on the basis of dry mass; in fact, a modest increase (+25%, nonsignificant) was seen in the ethanol-induced transgenics, compared with ethanol-induced WT. Sulfur and phosphorous levels were significantly increased (by 25% and 50%, respectively) in these same plants.

To rule out the possibility that *CpNifS* plants suffered from a defective chloroplast-import machinery, protein levels of the nuclear encoded protochlorophyllide reductase B and the small subunit of Rubisco were tested to determine whether their mature protein levels are reduced together with accumulation of precursors, as reported for chloroplast-import mutants (20, 21). Both proteins were present in mature size at equal levels in all plant types and treatments. Protochlorophyllide reductase B and the small subunit of Rubisco precursor-sized proteins did not accumulate, suggesting that protein import was not affected by *CpNifS* silencing. Finally, the abundance of two mitochondrial Fe-S proteins, Nad9 (a component of respiratory complex I) and biotin synthase, were not affected by *CpNifS* silencing (Fig. 5*D*). In summary, *CpNifS* silencing seems to specifically affect the maturation of Fe-S proteins in plastids.

Discussion

Silencing of *CpNifS* severely affected levels of chloroplastic Fe-S proteins and photosynthesis, and prolonged silencing resulted in a pleiotropic-stressed phenotype and eventually plant death. These results suggest that *CpNifS* is an essential protein that functions in plastid Fe-S cluster assembly and cannot be bypassed or complemented by MtNifS. When silencing was induced after the seedling stage, specific and reversible defects could be observed that yielded information about the functions of *CpNifS*. Silencing of *CpNifS* caused a defect in the accumulation of all eight chloroplastic Fe-S cluster proteins that were tested. The Fe-S proteins affected by *CpNifS* silencing together represent all five types of Fe-S clusters found in plastids, supporting the hypothesis that the cysteine desulfurase activity of CpNifS is required for the maturation of all Fe-S proteins in this organelle. Two mitochondrial Fe-S proteins were not affected, lending evidence that Fe-S cluster assembly in the mitochondria can operate independently of the chloroplastic cysteine desulfurase.

Fd did not exhibit any decrease when ethanol was initiated at week 3. However, Fd was absent when *CpNifS* silencing was induced with ethanol from germination, suggesting that holo-Fd is very stable once formed. In contrast to Fd, several other chloroplastic Fe-S proteins had decreased significantly 10 days after *CpNifS* silencing at week 3. These different delays in reduction of Fe-S proteins after *CpNifS* silencing may reflect protein stability or priority of Fe-S cofactor delivery.

The observed defects in photosynthetic electron transport and carbon fixation likely were a consequence of the lack of thylakoid Fe-S proteins, particularly in the cytochrome b_6f complex and PSI. Indeed, PSI function was severely compromised after *CpNifS* silencing. At the same time, PSII was only marginally affected in comparison, as evidenced by the presence of the D1 protein and functional heat dissipation, measured as nonphotochemical quenching of chlorophyll fluorescence (data not shown). A comparison of the Φ_{PSII} and Fv/Fm images suggests that Φ_{PSII} was affected before Fv/Fm, which may imply that damage to PSII could be a secondary consequence of a downstream defect in photosynthetic electron transport.

The altered chloroplast ultrastructure observed in *CpNifS*-silenced lines is reminiscent of the ultrastructure reported for an *APO1* mutant (22) that affects PSI accumulation, as well as a mutant in *Hcf101* and other mutations that affect PSI (23, 24). Therefore, the dilated stromal lamellae and absence of grana may be a consequence of a lack of PSI.

Mitochondria and chloroplasts originated from separate endosymbiotic events during the evolution of eukaryotes, and the two organelles have separate NifS-like proteins. It is likely that these two NifS-like proteins with their different properties each evolved to function optimally in their respective environments. The main function of mitochondria is to carry out the oxygen-consuming process of respiration, whereas chloroplasts perform the oxygen-generating process of photosynthesis. Thus, although both organelles contain an electron-transport chain that depends on Fe-S protein assembly, they contrast in redox conditions. Moreover, photosynthesis is known to produce reactive oxygen species, which can lead to oxidative stress. Fe-S cluster biosynthesis is particularly sensitive to oxygen. Therefore, it is not surprising that the chloroplastic NifS that has to operate under high-oxygen conditions is most similar to the bacterial SufS, which is thought to function under oxidative stress (3). MtNifS is most similar to bacterial IscS, the housekeeping NifS-like protein that is more sensitive to oxygen. Therefore, MtNifS likely would not function properly in an oxygen-producing compartment.

Another difference between the chloroplast and mitochondrion is that the chloroplast is the main site of cysteine synthesis in plant cells. It may be important to tightly control the cysteine desulfurase activity of the chloroplastic NifS, to avoid futile cycling. CpNifS may be particularly suited for the chloroplast because its cysteine desulfurase activity is extremely low in the absence of its activator CpSufE, in contrast to group I NifS-like proteins such as IscS and MtNifS (11). In summary, the two Fe-S cluster biosynthesizing machineries in the chloroplast and mitochondrion likely have different evolutionary origins and display properties that fit their function and environmental conditions.

After these two Fe-S biosynthesis machineries came together in the same plant cell, have they shared or transferred some of their functions? As shown here, *CpNifS* silencing is lethal in *Arabidopsis* and affects all five chloroplast Fe-S cluster types. Thus, MtNifS cannot complement the function of CpNifS in the biogenesis of any of these cluster types. At this point, it has not been reported whether MtNifS is essential as well, as was shown to be the case in yeast (25). In our studies, *CpNifS* silencing had no effects on mitochondrial Fe-S protein levels or respiration, suggesting that the mitochondrial Fe-S biogenesis machinery does not depend on CpNifS. Together, these results indicate that in plants, mitochondria and chloroplasts still have separate, essential cysteine desulfurases and Fe-S cluster assembly machineries.

Materials and Methods

Generation of *CpNifS*-Silencing Constructs and Induction. Standard cloning techniques were used to make the plant-transformation constructs and to generate transgenic *Arabidopsis thaliana*. For a detailed description of the cloning steps, see *SI Text, Supporting Information on the Cloning and Plant Transformation*. The primers

used in plasmid construction and verification are listed in SI Table 2. Ten constitutive RNAi lines and 11 inducible RNAi lines were obtained, which were selfed and propagated to homozygosity. Two of these lines were used for further functional characterization: *CpNifS-RNAi-6* and *CpNifS-RNAi-9* (denoted as *CpNifS-6* and *CpNifS-9*, respectively). To induce the RNAi construct, plants grown in soil were sprayed and soil-drenched every 4 days with a 2% ethanol solution, a concentration that was reported not to induce stress (26, 27); untreated control plants were sprayed with water. For RNAi induction on agar medium, plants were germinated on 0.5 strength Murashige and Skoog medium + 1% sucrose (28) solidified with 0.4% Agargel (Sigma) in 15-cm-diameter Petri dishes with 50 μl of 100% ethanol (or water for controls) placed in the center at the time of germination.

RT-PCR and Immunoblotting. The presence of transcripts in plants was detected by using RT-PCR (29). Protein extraction, SDS/PAGE, and immunoblot analysis were performed essentially as described (30). Leaf tissue for protein analysis was collected 10 days after induction. Antibodies for CpNifS (9), CpSufE (11), CpIscA (17), Fd and SiR (31), cytochrome *f*, light-harvesting complex of PSI, and PsaA/B (32), Fd-GOGAT and NiR (33), cytochrome b_6 and the Rieske subunit (34), PsaC and PsaD (35), protochlorophyllide reductase B and the small subunit of Rubisco (36), and the chloroplastic RRF (37) have been described. Specific antibodies for the D1 subunit of PSII and subunit A of ATP synthase were generous gifts from Alice Barkan (University of Oregon, Eugene) and Anna Sokolenko (Ludwig-Maximilians University, München, Germany), respectively. Specific antibodies for mitochondrial biotin synthase (38) and Nad9 (39) have been described. The intensity of mRNA and protein bands was quantified by using Image J imaging software [National Institutes of Health, Bethesda (http://rsb.info.nih.gov/ij)].

Electron Microscopy. Leaves were sampled from soil-grown plants 10 days after the start of ethanol treatment. Fixation and sectioning before analysis by transmission electron microscopy were performed as described (40).

Photosynthesis and Respiration Measurements. Chlorophyll content was assayed as described (41). Chlorophyll fluorescence images of Fv/Fm and Φ_{PSII} were captured from control and ethanol-treated dark-adapted soil-grown plants by using a Photon System Instruments imaging system (Photon System Instruments, Brno, Czech Republic). Default protocol settings were used with an actinic light intensity of 110 μE. A fluorescence monitoring system chlorophyll fluorometer (Hansatech, Cambridge, U.K.) was used for quantitative chlorophyll fluorescence analysis on detached, fully expanded leaves taken from dark-adapted plants. Fv/Fm, Φ_{PSII}, and ETR were calculated as described (14).

Photooxidation and dark-reduction kinetics of P700 (PSI) were measured in leaf disks by determining the light-induced absorbance change at 820 nm (ΔA_{820}) (16, 42) and at a saturating light intensity of 1600 μE, determined empirically. Carbon assimilation was assayed in detached leaves at 770 μE by using a Qubit Systems analyzer according to manufacturer instructions (Qubit Systems, Kingston, Ontario, Canada). Oxygen consumption by dark respiration was monitored in leaf tissue with a Hansatech LD2/3 leaf-disk O_2 electrode system maintained at 26°C.

Elemental Analysis, Enzyme Activity, and Statistics. Elemental composition was measured as described previously (43). Enzyme activity of NiR was measured according to ref. 44. All statistical analyses (ANOVA, *t* tests) were performed by using the Jmp-In software package (SAS Institute, Cary, NC).

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Supplementary information on the cloning and plant transformation:

The Expand polymerase (Roche, Pleasanton, CO) was used for PCR amplification and constructs were verified by DNA sequence analysis (CSU Macromolecular resources, Fort Collins, CO). The 3rd exon and intron were amplified from genomic DNA using primers 1 and 2 and cloned into pMOG18 (1) as an NcoI/BamH1 fragment. The resulting vector was linearized with *Bam*H1 and ligated to the *Bam*H1/*Bgl*2 digested inverted 3rd exon fragment obtained by PCR using primers 3 and 4. From the resulting vector the 1.6 kb *Eco*R1/*Hin*d3 fragment was subcloned into the binary vector pMOG23 (1) to generate a *CpNifS* RNAi construct under the control of the CaMV35S promoter. To generate an inducible construct the CpNifS exon-intron-exon construct was amplified using primers 5 and 6, digested with *Pst*1 and subcloned in pACN under control of the AlcA promoter (2, 3). The Hind3 fragment of the resulting vector was inserted into plant-binary vector pBinSRNACatN (Syngenta, Berkshire, UK), which contains the ethanol-inducible ALCR factor under the control of the CaMV35S promoter (2). *Agrobacterium tumefaciens* C58C1 was used to transform *Arabidopsis thaliana* (accession Col-0) using the floral dip method. Transgenic plants were selected by kanamycin resistance. Transformation was verified by PCR.

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S.I. Figure legends

S.I. Figure 6. Effect of CpNifS silencing on transcript levels of genes encoding Fe-S proteins. RNA blot analyses are shown for Rieske protein, GOGAT, sulfite reductase (SiR), and ascorbate peroxidase (APXI). 10 μg of RNA were loaded per lane. The 18S ribosomal RNA was visualized as a control. Numbers below bands represent the ratio of the intensity of the 18S band and the given mRNA product, in order to standardize the intensity of the bands.

S.I. Figure 7. Nitrite reductase (NiR) activity was reduced in CpNifS knockdown plants. $P < 0.05$ for 6-E and 9-E. All values represent the average and standard error of 5 independent measurements.

S.I. Figure 8. Time course of the depletion of CpNifS and Fe-S proteins upon ethanol induction. Wt and CpNifS-6 plants were soil grown and treated +/- ethanol for 10 days. Five of the youngest leaves from individual plants were removed on days 0, 1, 2, 4, 7, and 10 for protein analysis. Total protein extracts (25 μg protein) were separated by SDS-PAGE and blotted to nitrocellulose before immunostaining with indicated antibodies. Psa A-D, PSI subunits A-D; NiR, nitrite reductase; SiR, sulfite reductase; RRF, ribosomal recycling factor.

S.I. Table I. Elemental composition of leaf tissues. Shown are the means with the SE of the mean in parentheses (n=4). Lower case letters indicate significant differences ($P < 0.05$) between plant samples.

	WT-C	WT-E	CpNifS6-C	CpNifS6-E	CpNifS9-C	CpNifS9-E
Cu (μg/g DW)	14.5 (0.7)	12.2 (1.0)	13.7 (3.3)	16.1 (2.1)	14.0 (0.8)	13.2 (1.8)
Fe (μg/g DW)	55.5 (3.8)	45 (1.7)	41.2 (3.7)	56.1 (3.1)	49.9 (6.4)	53.6 (2.1)
Mg (mg/g DW)	9.0 (0.5)	7.7 (0.6)	7.8 (0.5)	8.8 (0.03)	8.1 (0.7)	9.6 (1.3)
Mn (μg/g DW)	147 (15) ^{b}	112 (11) ^{ab}	113 (15) ^{ab}	120 (2) ^{a}	129 (6) ^{a}	110 (2) ^{ab}
P (mg/g DW)	8.1 (0.4) ^{a}	8.2 (0.3) ^{a}	7.4 (0.3) ^{a}	12.2 (0.4) ^{b}	8.5 (0.6) ^{a}	12.8 (0.4) ^{b}
S (mg/g DW)	9.4 (0.8) ^{a}	10.1 (0.6) ^{a}	7.3 (0.6) ^{a}	12.3 (0.7) ^{b}	8.5 (0.4) ^{a}	12.8 (0.3) ^{b}
Zn (μg/g DW)	256 (13)	262 (9)	248 (16)	271 (24)	251 (11)	222 (25)

S.I. Table II: Primers used. Primer sequences are given starting from the 5' end and with indicated restriction sites underlined. CpNifS sequence is in capitals.

#1: catgccatggGTTATACTTACAGTTGCTGAACAT (*Nco*1)

#2: cgcggatccCAATCTCTTCAATAGGAAGAGAAG (*Bam*H1)

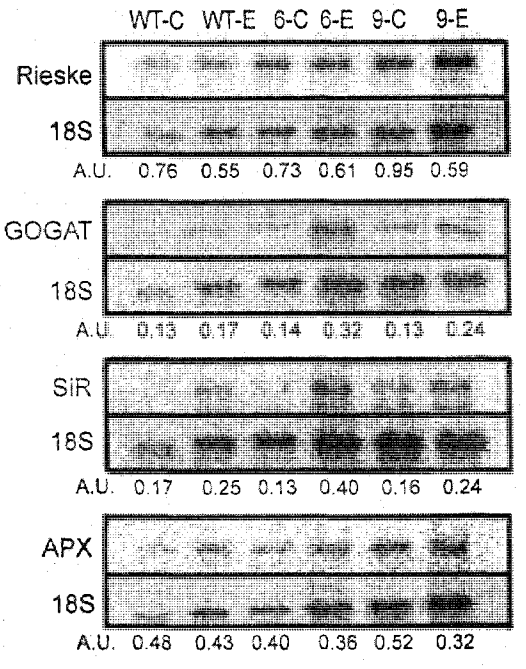
#3: cgcggatccGTTATACTTACAGTTGCTGAACAT (*Bam*H1)

#4: ggaagatctCAAGAACATTAGAAACATGATGAAC (*Bgl*2)

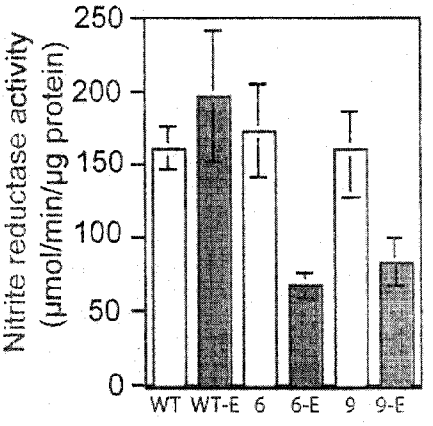
#5: ctgcagcaaatacttccaccatggGTTA (*Pst*1)

#6: ctgcagGTTATACTTACAGTTGCTGAACAT (*Pst*1)

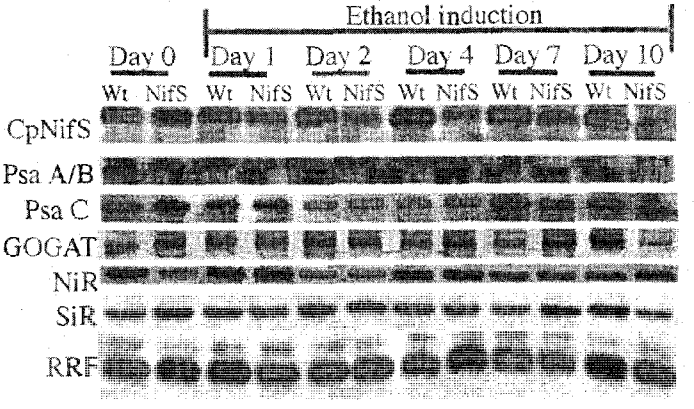
S.I. Figure 6


WT-C WT-E 6-C 6-E 9-C 9-E
Rieske
18S
A.U. 0.76 0.55 0.73 0.61 0.95 0.59
GOGAT
18S
A.U. 0.13 0.17 0.14 0.32 0.13 0.24
SiR
18S
A.U. 0.17 0.25 0.13 0.40 0.16 0.24
APX
18S
A.U. 0.48 0.43 0.40 0.36 0.52 0.32

S.I. Figure 7


Nitrite reductase activity
(µmol/min/µg protein)
250
200
150
100
50
0
WT
WT-E
6
6-E
9
9-E

S.I. Figure 8


Ethanol induction
Day 0
Day 1
Day 2
Day 4
Day 7
Day 10
Wt NifS Wt NifS Wt NifS Wt NifS Wt NifS Wt NifS
CpNifS
Psa A/B
Psa C
GOGAT
NiR
SiR
RRF

Chapter 6 - Summarizing discussion and broader impacts of the dissertation

As reviewed in chapter 2, NifS-like proteins are a conserved group of proteins that can cleave the sulfur-containing amino acid cysteine in alanine and elemental sulfur (S). In analogy, it can cleave selenocysteine (SeCys) into alanine and selenium (Se). In yeast and bacteria, NifS-like proteins are essential for survival because they provide the S for FeS clusters, a prosthetic group that is inserted into various FeS proteins that have a role in electron transfer. Furthermore, NifS-proteins are an essential part of Se metabolism in organisms that require this trace element.

Chapter 3 described how overexpression of CpNifS enhanced Se tolerance and accumulation in Arabidopsis plants growing on selenate. These results could be explained by the decreased amount of Se in protein in CpNifS overexpressing plants. One of the questions that remains is: where does the elemental Se go once it is cleaved from SeCys? It is possible that it is safely sequestered in the plastid, or perhaps conjugated to phytochelatin or glutathione, like sulfide can be. In this context it is interesting to note that overexpression of a mouse SL in the chloroplast was shown to be toxic to Arabidopsis, presumably because the elemental Se produced interfered with FeS cluster formation in this compartment (Pilon et al., 2002). It is possible that the endogenous SL activity of CpNifS is modulated in a way that renders its Se product harmless, e.g. via interaction with a plastidic partner protein. Future experiments using radioactive Se may help determine the fate of elemental Se once it is released from CpNifS.

Since CpNifS overexpressing Arabidopsis transgenics showed enhanced Se tolerance and accumulation, overexpression of CpNifS in other plant species may help further the field of phytoremediation. Potential plant species for this purpose could be Indian Mustard for terrestrial conditions, and Spartina or poplar for wetland conditions. Field trials with various other transgenics have already shown the potential of plant biotechnology to create plants that are better able to accumulate Se from polluted soils (Banuelos et al., 2005, 2007). Before any of these transgenics are implemented for phytoremediation, it should be determined that these plants are safe for the environment,

and that the risk of escaped pollen or horizontal gene transfer remains very low. Placing the CpNifS overexpressing construct into the chloroplast genome may help alleviate the latter concern. The other issue is whether these transgenic organisms that accumulate substantial levels of Se in the field are a threat to wildlife and public health. These concerns may be alleviated if it can be demonstrated that the plants volatilize most of the accumulated Se, rather than return their tissue Se to the soil Se pool during the mineralization process in the Fall and Winter months. Ecological studies on the effects of Se on plant-herbivore and plant-microbe interactions, and the movement of Se into the local ecosystem, may also help assess the risks associated with the use of plants for environmental cleanup of Se.

The transcriptome study described in Chapter 4 identified many Se-responsive genes in Arabidopsis. It is possible that some of these genes may further aid the applied fields of phytoremediation. Future experiments may include ordering seeds that harbor a T-DNA insertion of a few carefully selected Se-responsive genes, and determining if there is a phenotype in plants when a particular gene is knocked-out. Interestingly, CpNifS was not identified as one of the 1,300 Se-responsive genes that were identified in root and shoot tissue combined, which suggests that its true function may not be to confer protection against Se toxicity.

Indeed, it is more likely that the function of CpNifS is to release S from cysteine for the incorporation into FeS clusters. As revealed in Chapter 5, knockdown of CpNifS decreased nitrite reductase activity and the level of electron transport in the light reactions, as well as photosynthetic CO_2 uptake. These processes depend on chloroplastic FeS proteins, and indeed we observed a decrease or absence of all the chloroplastic FeS proteins that were tested. Together these observations explain the observed chlorotic phenotype of the CpNifS knockout plants, and their eventual death. The mitochondrial FeS-dependent process of respiration was not affected by a knockdown of CpNifS, nor were levels of mitochondrial FeS proteins. The observation that knockout of CpNifS is lethal shows that MtNifS cannot rescue plants that have drastically reduced levels of CpNifS. Thus, CpNifS appears to be responsible for providing the S for all plastidic FeS proteins, but not for FeS proteins in the mitochondria. Preliminary results from

collaboration with Janneke Balk (Cambridge, UK) indicates that the CpNifS knockouts are also not impaired in cytosolic FeS cluster formation (unpublished results).

In view of the evidence that CpNifS is essential for FeS clusters, the idea that CpNifS overexpression may increase levels of FeS proteins and thus photosynthesis was worth considering. Also, it would be beneficial if the transgenics would contain higher Fe levels, since a significant portion of the human population does not receive enough Fe from their diet. I questioned if overexpressing and knocking-down CpNifS protein would affect the amount of Fe levels in Arabidopsis. However, neither overexpression nor knockdown of CpNifS significantly increased or reduced Fe levels. Thus, there is no indication of a role for CpNifS in regulating the Fe status in plants, and no realistic prospect of using plants that overexpress CpNifS for the use of Fe-fortified foods.

The use of an inducible promoter was paramount to the success of the knockout study described in Chapter 5. Before using this approach, other approaches had already been tested and proven unsuccessful. The one available CpNifS T-DNA line showed no decrease in CpNifS expression. A subsequent antisense transgenic approach also did not lead to decreased CpNifS expression. This prompted the use of RNAi to knock down CpNifS levels. Constitutive CpNifS-RNAi was not stable in plants; using this approach I obtained transgenic plants that either had negligible knockdown of CpNifS or very sick plants that could not survive three days past germination. These observations suggest that CpNifS may be essential for survival, and that FeS clusters are required during early events in the plant life cycle. The use of an ethanol-inducible promoter to express the RNAi-CpNifS construct produced plants that can be considered conditional mutants. Conditional mutants allow a researcher to determine at what developmental stage a transgene or construct is expressed in an organism. Stated another way, a conditional mutant allows the researcher to determine when a normally-appearing "wildtype" organism becomes a mutant or transgenic. In conclusion, the ethanol-inducible system employed for this dissertation represents a very valuable tool for plant researchers to (a) study essential genes, (b) regulate the expression of exogenous genes, and (c) explore how biotechnology can be used to meet some of the challenges of society. For example, an inducible system can be envisioned to regulate at what developmental stage a bacterial

pectinase or fungal cellulase is expressed in plants for the advancement of cellulose-derived biofuels.

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Appendix

I. Arabidopsis plants overexpressing CpNifS do not have higher enzymatic activity.

CpNifS can catalyze the reaction of cysteine into alanine and elemental sulfur, as well as selenocysteine into alanine and elemental selenium. Thus, CpNifS is described as having cysteine desulfurase (CysD) and selenocysteine lyase (SL) activity. Overexpression of CpNifS roughly increased mRNA levels 40-fold and protein levels nearly 25-fold. Plants overexpressing CpNifS (p38 and p55) had increased plant tolerance to and accumulation of selenium, and a decreased incorporation of selenium in protein. Interestingly, the transgenic plants did not have higher SL activity compared to the WT plants.

It was considered important to demonstrate higher SL activity in NifS overexpressing plants to help explain their improved tolerance to selenium. Therefore, different conditions for the SL activity assay were adapted based on earlier published results (Esaki et al., 1982, Garifullina et al., 2003). The temperature, salt, and pH of the reaction were manipulated, and SL activity compared in CpNifS overexpressing plants and WT; Arabidopsis plants overexpressing a mouse selenocysteine lyase were used as a positive control (Pilon et al., 2003). Despite the attempts to adapt the SL activity assay , transgenic and WT plants exhibited roughly the same SL activity (Figure 1A). Desalting resulted in lower activity levels. There was no difference in activity at 25 and 37 degrees C, and the reaction only worked at a pH of 7.5 and not at pH 5.5 or 8.5 (data not shown). SL activity was 9-fold higher in transgenic plants with the mouse SL protein compared to WT plants.

Since SL activity was not higher in p38 and p55, I attempted to see if these plants had higher CysD activity. This enzyme activity assay was performed essentially as described (Outten et al., 2003). Lines p38 and p55 also nearly had the same CysD activity compared to WT plants, both in total plant homogenate and in a stromal fraction (Figure 1B,C), even after the addition of SufE, which is know to stimulate CysD activity (Ye et al., 2006)

In view of the overexpression of CpNifS protein, and the fact that NifS overexpression increased tolerance to selenate and decreased the amount of Se in protein, it is inexplicable that the transgenic plants did not exhibit higher SL activity. The CpNifS gene construct in line p55 was resequenced, and I can rule out that a point mutation had been introduced into the transgenic plants. It is possible that, for some reason, SL activity in the transgenic plants is higher than in WT *in vivo* but not *in vitro*.

II. Arabidopsis plants overexpressing CpNifS do not have higher photosynthetic efficiency or perform better during sulfur and iron starvation.

As described in Chapter 2, CpNifS overexpression enhances selenate tolerance and selenium accumulation. Next I questioned if protection from selenate conferred improved photosynthetic performance when transgenic plants were grown on selenate. Furthermore, since CpNifS has a role in Fe-S proteins necessary for photosynthesis, as described in Chapter 4, it was hypothesized that overexpression of CpNifS may lead to improved photosynthetic performance under standard growth conditions.

WT, p38, and p55 plants were grown in soil for three weeks, at which point 50 mL of 40uM selenate was fed to the plants every 3 days for 2 weeks. Control plants received water. A fluorescence monitoring system (Hansatech, Cambridge, UK) was then used for chlorophyll fluorescence analysis on detached leaves (Van Hoewyk, et al., 2007).

Photosynthetic efficiency (as measured by Fv/Fm and Φ_{PSII}, which is used to estimate the electron transport rate) was not improved in the transgenic plants compared to the wildtype plants, either with or without selenate (Figure 2). Plants were then subjected to light stress by placing the plants closer (<10 cm) to additional light sources, which reduced the Φ_{PSII} by roughly 60%. Again, there was no difference in the Φ_{PSII} between WT and transgenic plants when subjected to high light stress. These data suggest that overexpression of CpNifS does not increase photosynthetic performance either with or without selenate, or during high light stress.

Chapter 4 revealed that depletion of CpNifS drastically reduced levels of Fe-S proteins, as well as its activator, SufE. I thought it would be worthwhile to determine if

CpNifS overexpression resulted in higher levels of chloroplastic FeS proteins, and better growth during Fe or S starvation. I hypothesized that:

(1) overexpression of CpNifS would increase protein levels of FeS proteins,

(2) transgenic plants would grow better under Fe or S starvation, and

(3) levels of Fe-S proteins would be higher in transgenic plants subjected to Fe or S starvation.

For the S starvation experiment, WT and p55 plants were grown on Hoagland's media containing 3.7 mM, 1 mM, 50 uM, or 10 uM sulfate. Complete Hoagland's media contains roughly 1 mM sulfate. Thus, in addition to the sulfate concentration that is regularly found in Hoagland's media, WT and p55 plants were subjected to sulfate levels nearly 4-fold higher (3.7 mM) as well as 20-fold and 100-fold lower (50 uM and 10 uM). For the Fe starvation experiment, WT and p55 plants were grown on 0.5 MS media with or without 0.1 M and 1.0 M ferrozine, which is an Fe chelator. Plants were grown on horizontal plates for 2 weeks, at which time the plants were harvested, proteins were extracted, and SDS-PAGE was performed. Antibodies against SufE and two FeS proteins, SiR (sulfite reductase) and ferredoxin-dependent GOGAT (which is involved in N assimilation), were used to determine the levels of these proteins in the samples.

Plants growing on excess S (3.7 mM sulfate) did not grow any better than plants growing on complete Hoagland's media. In contrast, S and Fe limitation greatly reduced plant productivity and growth compared to plants growing on complete Hoagland's media and 0.5-strength MS media. CpNifS overexpressing plants (line p55) did not grow better than WT plants under any of these treatments (results not shown).

In general, the protein levels of SufE, SiR, and Fd-GOGAT did not differ between wildtype and transgenic plants when grown on complete Hoagland's media and 0.5MS media, nor when plants were grown on excessive S or on limited S and Fe (Figure 3). The one notable exception may be SiR in plants grown on 1 mM ferrozine: under these conditions of Fe limitation SiR decreases, but apparently less so in p55 compared to WT.

In conclusion, overexpression of CpNifS does not have a clear effect on the levels of the two FeS proteins tested, either on control media or during S or Fe starvation (except that SiR decreased during extreme Fe starvation). Additionally, SufE, SiR and Fd-GOGAT were similarly expressed, regardless of S or Fe starvation, although it should

be noted that SiR decreased slightly during Fe starvation. Furthermore, the NifS overexpressing line p55 did not grow any better on horizontal plates during S and Fe limitation. In Chapter 2, overexpression of CpNifS was shown to not widely affect the transcriptome of Arabidopsis plants either on control media or selenate. Data from the S and Fe starvation experiment complement the microarray data in Chapter 2, and suggest that overexpression of CpNifS does not result in pleiotropic effects and in the ability of CpNifS transgenic plants to withstand a variety of stresses. Thus, there is no indication that overexpression of CpNifS confers protection against any stress other than selenate stress.

III. NifS proteins in *Plasmodium falciparum* and *Trypanosoma brucei*

Plasmodium falciparum, the causative agent of malaria, and other apicomplexan parasites, contain apicoplasts (Ellis et al., 2001). Apicoplasts are non-photosynthetic plastids derived from cyanobacteria during an endosymbiotic event. *P. falciparum* contains a mtNifS-like protein, as well as another nuclear-encoded NifS-like protein that is targeted to the apicoplast (Ellis et al., 2001) and designated acNifS. An amino acid analysis using the software program ClustalW2 (Larkin et al., 2007) reveals that acNifS is 27% similar to Arabidopsis cpNifS and is 30% similar to *E.coli* SufS and the rice cpNifS homologue, all of which are Group II NifS proteins (Figure 4). Despite the relatively low homology of acNifS to other Group II NifS proteins, acNifS indeed belongs to this group based on a conserved cysteine (amino acid #496) (Figure 4).

Trypanosoma brucei, a protist that causes sleeping sickness, is known to contain two NifS-like proteins. One protein is localized to the cytosol, where it is thought to contain SL activity, and the mitochondrial protein is expected to possess SL activity (Pavel Poliak and Julius Luke, personal communication). The mitochondrial NifS protein in *T. brucei* has high amino acid similarity to the Arabidopsis mtNifS protein (59%) and *E.coli* IscS (56%). The cytosolic NifS-like protein in *T. brucei* is 30% homologous to AtMtNifS and IscS, but lacks homology to the NifS domain of cytosolic ABA3 or cpNifS in Arabidopsis (Figure 5).

Inducible RNAi lines for both NifS proteins in *T. brucei* (TbCtyNifS and TbMtNifS) were constructed in the lab of Julius Luke (Czech Republic). WT as well as induced and non-induced RNAi lines were grown and harvested. Samples of mitochondrial and cytosolic protein fractions from the different lines were sent on dry ice to CSU, where I performed SL and CysD activity assays on the samples.

Briefly, mitochondrial SL activity decreased 30-80% in both TbCtyNifS and TbMtNifS RNAi lines; this also includes the non-induced lines, possibly indicating that the inducible promoter is leaky (Figure 6). However, it should be mentioned that mRNA levels of the RNAi lines have not yet been measured (Julius Luke, personal communication). SL activity in the cytosol did not change as drastically as it did in the mitochondria. Mitochondrial CysD activity in the induced RNAi lines for both TbCtyNifS and TbMtNifS decreased compared to the non-induced controls. In contrast, cytosolic CysD activity increased in the induced mutants, perhaps trying to compensate for the lowered CysD activity in the mitochondria. However, this interpretation should again be approached with caution, since there is no evidence that FeS clusters made in the cytosol can be transported to the mitochondria.

In summary, the TbMtNifS RNAi lines did exhibit less mitochondrial CysD activity as expected, but surprisingly the TbCytNifS RNAi lines did not have less SL activity in the cytosolic fractions. Again, a more detailed characterization of the *T. brucei* RNAi lines is warranted, including analysis of the mRNA levels of the NifS genes.

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Figure 1: SL and CysD activity in CpNifS overexpressing plants. (A) SL activity in WT, p38, p55, and plants overexpressing a mouse SL protein. Desalted reactions were also carried out. (B) CysD activity in crude plant extracts with and without the addition of 15ug of SufE. Pure SufE is also shown as a negative control. (C) CysD activity in stromal fractions of isolatedchloroplasts with and without the addition of 15 ug of SufE.

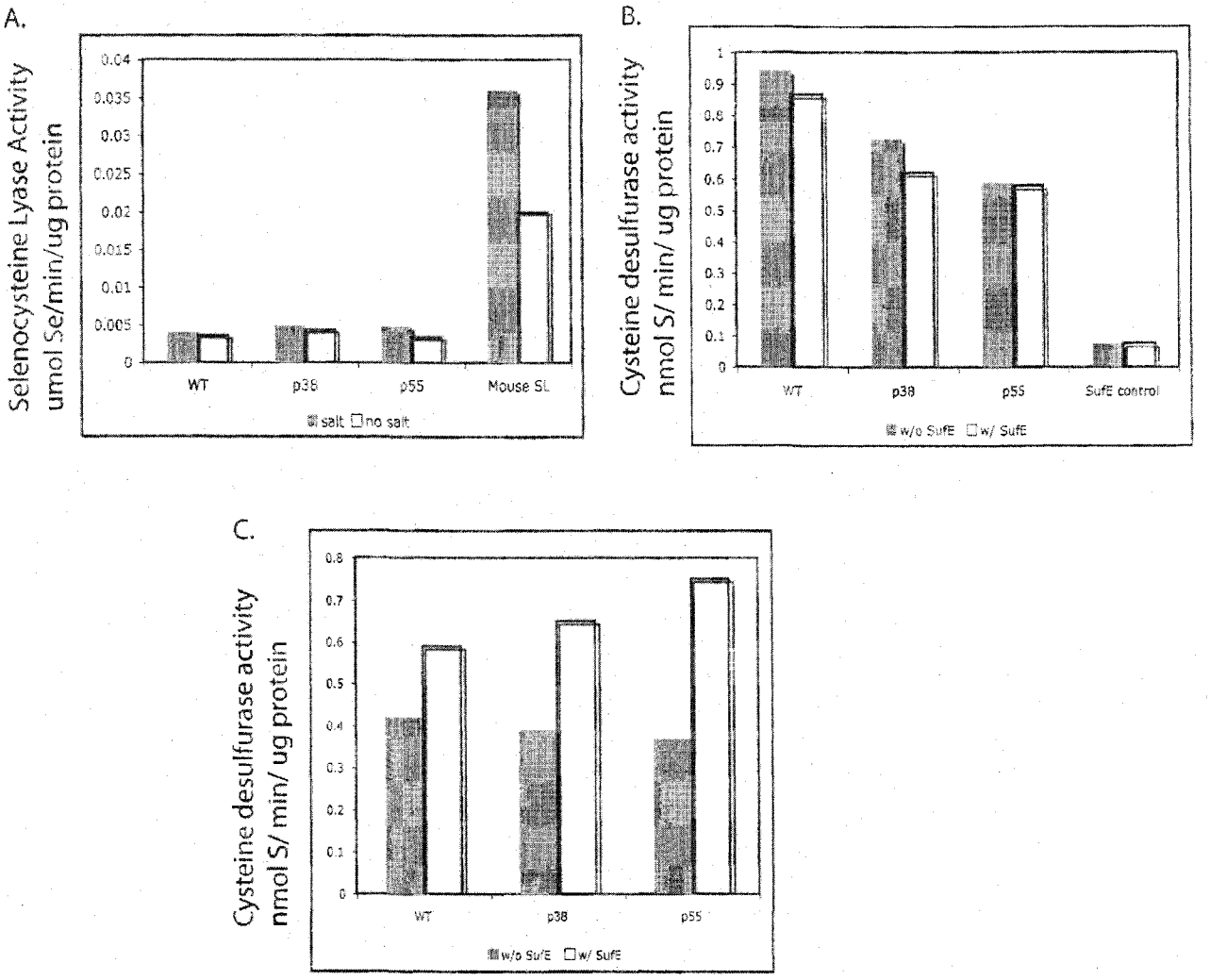


Figure 2. Chlorophyll fluorescene analysis. Plants were grown in soil and treated with or without selenium (Se). Fv/Fm in dark-treated leaves (top). Electron transport rate at low and high light (bottom). Three replicates were used for each experiment.

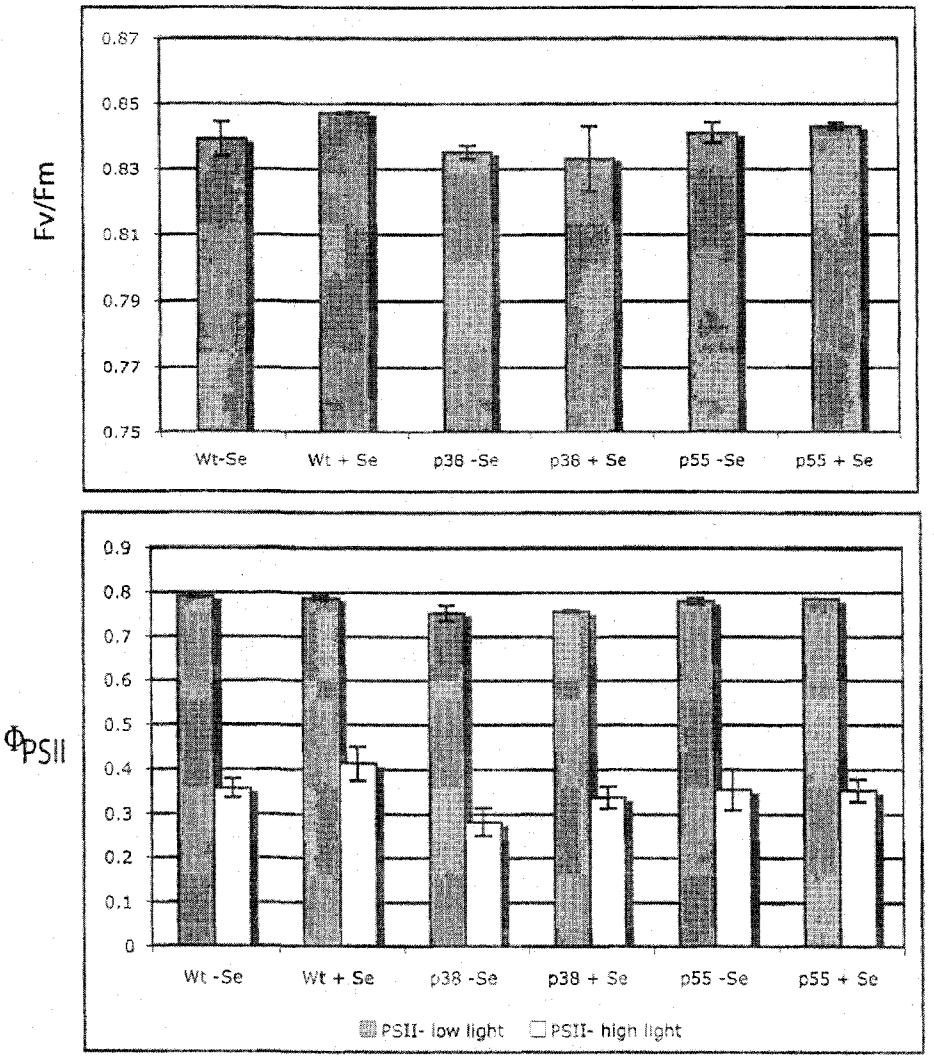


Figure 3. Effects of CpNifS overexpression on protein levels during sulate (S) and iron (Fe) starvation and excessive S. 1mM S is considered standard in complete Hoagland's media. Ferrozine is an Fe chelator. SiR, sulfite reducatse; Fd-GOGAT, ferredoxin dependant GOGAT.

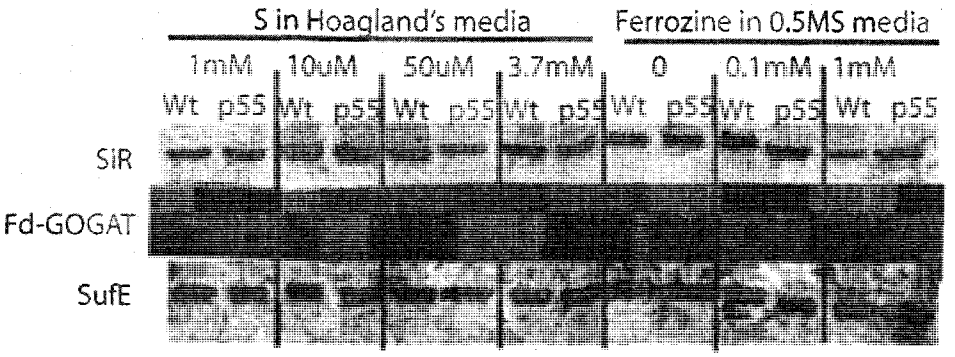
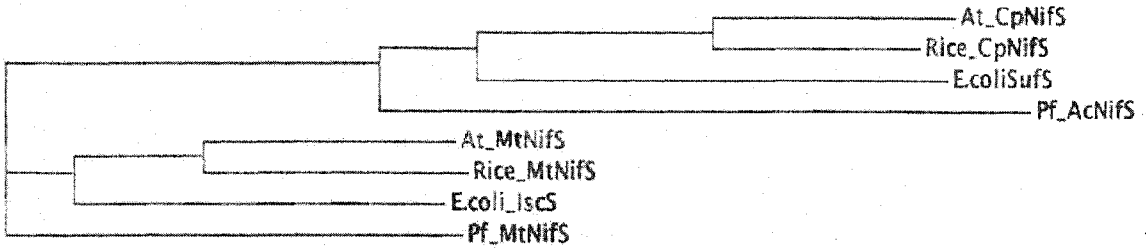


Figure 4. Amino acid alignment of mitchondrial (Mt), chloroplastic (Cp), and apicoplast (Ac) NifS-like proteins in Arabidopsis (At) , E.coli, Rice, and Plasmodium falciparum (Pf). Note SufS and IscS are E.coli homologues to chloroplast and mitochondrion NifS proteins. The amino acid aligment score (top) and phylogram (bottom) are depicted. The alignment of the proteins is provided on the next page.

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	At_CpNifS	463	2	At_MtNifS	453	18
1	At_CpNifS	463	3	Pf_AcNifS	546	27
1	At_CpNifS	463	4	Pf_MtNifS	553	15
1	At_CpNifS	463	5	E.coliSufS	406	47
1	At_CpNifS	463	6	E.coli_IscS	404	22
1	At_CpNifS	463	7	Rice_CpNifS	375	73
1	At_CpNifS	463	8	Rice_MtNifS	458	16
2	At_MtNifS	453	3	Pf_AcNifS	546	15
2	At_MtNifS	453	4	Pf_MtNifS	553	47
2	At_MtNifS	453	5	E.coliSufS	406	19
2	At_MtNifS	453	6	E.coli_IscS	404	55
2	At_MtNifS	453	7	Rice_CpNifS	375	22
2	At_MtNifS	453	8	Rice_MtNifS	458	70
3	Pf_AcNifS	546	4	Pf_MtNifS	553	15
3	Pf_AcNifS	546	5	E.coliSufS	406	30
3	Pf_AcNifS	546	6	E.coli_IscS	404	12
3	Pf_AcNifS	546	7	Rice_CpNifS	375	30
3	Pf_AcNifS	546	8	Rice_MtNifS	458	13
4	Pf_MtNifS	553	5	E.coliSufS	406	17
4	Pf_MtNifS	553	6	E.coli_IscS	404	48
4	Pf_MtNifS	553	7	Rice_CpNifS	375	18
4	Pf_MtNifS	553	8	Rice_MtNifS	458	45
5	E.coliSufS	406	6	E.coli_IscS	404	19
5	E.coliSufS	406	7	Rice_CpNifS	375	44
5	E.coliSufS	406	8	Rice_MtNifS	458	18
6	E.coli_IscS	404	7	Rice_CpNifS	375	24
6	E.coli_IscS	404	8	Rice_MtNifS	458	56
7	Rice_CpNifS	375	8	Rice_MtNifS	458	21



CLUSTAL W 2.0 multiple sequence alignment

```
At_MtNifS                   ----------------------------------------------------MASKVI  6
Rice_MtNifS                 ----------------------------------------------------MALSRRLL  8
E.coli_IscS                 ------------------------------------------------------------
Pf_MtNifS                   MKFLQIIKHLKLQNKKNALDNFVNCRTYEHISNINKLFLNNFSSTKEHSEHGQVKHENFL 60
At_CpNifS                   -----------------------------------MEGVAMKLPSFPNAISIGHRS 21
Rice_CpNifS                 ------------------------------------------------------------
E.coliSufS                  ------------------------------------------------------------
Pf_AcNifS                   ---------------------------MLRGPRCLYIYLFFVFLPFSFCYIRNNDNRF 31

At_MtNifS                   SATIRRTLT---KPHGTFSRCRYLSTAAAATEVNYED---------------------- 40
Rice_MtNifS                 PLLLRRGSTPSLSPARALSTAAVTADAPAAAAAAAEE---------------------- 45
E.coli_IscS                 ------------------------------------------------------------
Pf_MtNifS                   NSTLKYEENSQNGSTNNLKNGKYNMYVSEGNVNINEEKYKDNNISSNNTQYNNNSSNSGS 120
At_CpNifS                   FSRVRCSSSLSVCSAAAASSATISTDSESVSLGHRVR---------------------- 58
Rice_CpNifS                 ------------------------------------------------------------
E.coliSufS                  ----------------------------MIFSVDKVR---------------------- 9
Pf_AcNifS                   VYIVKSIRKGPNIKLRLTKDEKPNIDNHIIDYFKNVR---------------------- 68

At_MtNifS                   ------------ESIMMKGVRISGRPLYLDMQATTPIDPRVFDAMNASQIHE--YGNPHS 86
Rice_MtNifS                 ------------EAMTIKGVRISGRPLYMDMQATTPVDPRVLDAMLPFYLSR--YGNPHS 91
E.coli_IscS                 -----------------MK------LPIYLDYSATTPVDPRVAEKMMQFMTMDGTFGNPAS 38
Pf_MtNifS                   LNDEGPLWKEHIDDVVNENKKKKMNRFYLDSQATTMIDPRVLDKMLPYMTYI--YGNAHS 178
At_CpNifS                   -----------KDFRILHQEVNGSKLVYLDSAATSQKPAAVLDALQNYYEFY--NSNVHR 105
Rice_CpNifS                 ----------------------------------------MKTLDEYYRFY--NSNVHR 17
E.coliSufS                  -----------ADFPVLSREVNGLPLAYLDSAASAQKPSQVIDAEAEFYRHG--YAAVHR 56
Pf_AcNifS                   --------------EHFPFFKENKSLIYFDSAATTHKPSCVIEKMSEFYKKE--NSNIHR 112
                                                                    .          .

At_MtNifS                   RTHLYGWEAENAVENARNQVAKLIEASP-KEIVFVSGATEANNMAVKGVMHFYKDTKK-- 143
Rice_MtNifS                 RTHLYGWESDAAVEEARARVASLVGADP-REIFFTSGATECNNIAVKGVMRFYRDRRR-- 148
E.coli_IscS                 RSHRFGWQAEEAVDIARNQIADLVGADP-REIVFTSGATESDNLAIKGAANFYQKKGK-- 95
Pf_MtNifS                   RNHFFGWESEKAVEDARTNLLNLINGKNNKEIIFTSGATESNNLALIGICTYYNKLNKQK 238
At_CpNifS                   GIHYLSAKATDEFELARKKVARFINASDSREIVFTRNATEAINLVAYSWGLSNLKPGD-- 163
Rice_CpNifS                 GIHVLSAKATDAYESARTKVANFVNAANSREIVFTRNATEAINLVAYSWGMSNLKQGD-- 75
E.coliSufS                  GIHTLSAQATEKMENVRKRASLFINARSAEELVFVRGTTEGINLVANSWGNSNVRAGD-- 114
Pf_AcNifS                   GIYKLSHNATNNYEKVRETIKEYINCEKNDNIIFTNGSTYGLNVVCKMMIEEIIKKEED- 171
                              :  . ::    : .*      :      ::.*. .:*   *:.

At_MtNifS                   -HVITTQTEHKCVLDSCRHLQQEG-FEVTYLPVKTDGLVDLEMLREAIRPDTGLVSIMAV 201
Rice_MtNifS                 -HVVTTQTEHKCVLDSCRYLQQEG-FEVTYLPVRPDGLVDVAQLADAIRPDTGLVSVMAV 206
E.coli_IscS                 -HIITSKTEHKAVLDTCRQLEREG-FEVTYLAPQRNGIIDLKELEAAMRDDTILVSIMHV 153
Pf_MtNifS                   NHIITSQIEHKCILQTCRFLQTKG-FEVTYLKPDTNGLVKLDDIKNSIKDNTIMASFIPV 297
At_CpNifS                   EVILTVAEHHSCIVPWQIVSQKTG-AVLKFVTLNEDEVPDINKLRELISPKTKLVAVHHV 222
Rice_CpNifS                 EIVLTIAEHHSAIVPWQFVSQKTG-ATLKYVGLTKEGVPDIEQLKGLLSNKTKIVVVHHV 134
E.coliSufS                  NIIISQMEHHANIVPWQMLCARVG-AELRVIPLNPDGTLQLETLPTLFDEKTRLLAITHV 173
Pf_AcNifS                   EIYLSYMEHHSNIIPWQEYINKEKKGRIKYVPLNKSGYINIKKLISNMNINTKVISICHA 231
                               ::   .*  ::             :  :    .   .:  :   :  .* :  .  .

At_MtNifS                   NNEIGVVQPMEEIGMICKEHN--VPFHTDAAQAIGKIPVDVKKWN-----VALMSMSAHK 254
Rice_MtNifS                 NNEIGVVQPLEEIGRICKEKG--VPFHTDAAQALGKIPIDVNQMG-----IGLMSLSAHK 259
E.coli_IscS                 NNEIGVVQDIAAIGEMCRARG--IIYHVDATQSVGKLPIDLSQLK-----VDLMSFSGHK 206
Pf_MtNifS                   NNEIGVIQDIENIGNLCKEKN--ILFHTDASQAAGKVPIDVQKMN-----IDLMSMSGHK 350
At_CpNifS                   SNVLASSLPIEEIVVWAHDVG--AKVLVDACQSVPHMVVDVQKLN-----ADFLVASSHK 275
Rice_CpNifS                 SNVLGSMLPIEDIVTWSNRIG--AKVLVDACQSVPHMPVDVQRLG-----ADFLVASSHK 187
E.coliSufS                  SNVLGTENPLAEMITLAHQHG--AKVLVDGAQAVMHHPVDVQALD-----CDFYVFSGHK 226
Pf_AcNifS                   SNVIGNIQNIEKIIKKIKNVYPHIIIIDASQSFAHIKYDIKKMKKNKSCPDILITSGHK 291
                            .* :.    :  :    .          *. *:  :   *:.          :   *.**

At_MtNifS                   IYGPKGVGALYVRRR-PRIRLEPLMNGGGQERGLRSGTGATQQIVGFGAACELAMKEMEY 313
Rice_MtNifS                 IYGPKGVGALYLRRR-PRIRVEPQMSGGGQERGIRSGTVPTPLVVGFGAACEIAAKEMDY 318
E.coli_IscS                 IYGPKGIGALYVRRK-PRVRIEAQMHGGGHERGMRSGTLPVHQIVGMGEAYRIAKEEMAT 265
Pf_MtNifS                   LYGPKGIGALYIKRKKPNIRLNALIHGGGQERGLRSGTLPTHLIVGFGEAAKVCSLEMNR 410
At_CpNifS                   MCGPTGIGFLYGKSD--LLHSMPPFLGGGE---MISDVFLDH-STYAEPPSRFEAGTPAI 329
Rice_CpNifS                 MCGPTGVGFLHGKFD--LLSSMEPFLGGGE---MIADVFQDK-STYAEPPSRFEAGTPAI 241
E.coliSufS                  LYGPTGIGILYVKEA--LLQEMPPWEGGGS---MIATVSLSEGTTWTKAPWRFEAGTPNT 281
Pf_AcNifS                   FCASLGTGFIFINKELSSKYKFKPLLYGSN---IITNVSKYKSKFVTSLSELLETGTQNI 348
                            : .. * * :. .             *.    : : .          .  .
```

```
At_MtNifS                       DEKWIKGLQERLLNGVREKLDGVVVN---GSMDSRYVGNLNLSFAYVEGESLLMGLKEVA 370
Rice_MtNifS                     DHRRASVLQQRLLDGIRGQVDDIVIN---GSMEHRYPGNLNLSFAYVEGESLLMGLKEVA 375
E.coli_IscS                     EMERLRGLRNRLWNGIKD-IEEVYLN---GDLEHGAPNILNVSFNYVEGESLIMALKDLA 321
Pf_MtNifS                       DEKKVRYFFNYVKDYLTKHLDYIVFN---GCQINRYYGNMNISFLFVEGESLLMSLNEIA 467
At_CpNifS                       GEAIALGAAVDYLSGIGMPKIHEYEV---EIGKYLYEKLSSLPDVRIYGPRPSESVHRGA 386
Rice_CpNifS                     GEAIGLGTAIDYLSQIGMQKIHEYEN---ELATYLYESLIAVPNVRIYGPAPCQTVHRAP 298
E.coliSufS                      GGIIGLGAALEYVSALGLNNIAEYEQ---NLMHYALSQLESVPDLTLYGPQ-----NRLG 333
Pf_AcNifS                       PGILSMGISLEFFKKINWNYVYQYEMYLYDLFIYYMNKYMKNHFVQLPNLNLSYKKENIN 408
                                             . :                                : .       .

At_MtNifS                       VSSGSACTSASLEPSYVLRALGVD---------------------------------- 394
Rice_MtNifS                     VSSGSACTSASLEPSYVLRALGVE---------------------------------- 399
E.coli_IscS                     VSSGSACTSASLEPSYVLRALGLN---------------------------------- 345
Pf_MtNifS                       LSSGSACTSSTLEPSYVLRSIGIS---------------------------------- 491
At_CpNifS                       LCS---FNVEGLHPTDLATFLDQQ---------------------------------- 407
Rice_CpNifS                     LCS---FNVENVHPTDIAEILDLQ---------------------------------- 319
E.coliSufS                      VIA---FNLGKHHAYDVGSFLDN---------------------------------- 353
Pf_AcNifS                       YKSHMQTHPPVHKYNDEQNFTNDHNITQSKQTKSIHSQHDTFKIYTHDTRKYGLKKIGIL 468
                                  :          .         .

At_MtNifS                       ------------------EDMAHTSIRFGIGRFTT-KEEIDKAVELTVKQVEKLREMSPL 435
Rice_MtNifS                     ------------------EDMAHTSIRFGIGRFTT-EEEVDRAIELTVHQVKKLRDMSPL 440
E.coli_IscS                     ------------------DELAHSSIRFSLGRFTT-EEEIDYTIELVRKSIGRLRDLSPL 386
Pf_MtNifS                       ------------------EDIAHTSIRIGFNRFTT-FFEVQQLCINLVKSVERLRSISPL 532
At_CpNifS                       ------------------HGVAIRSGHHCAQPLHR-YLGVNASARASLYFYNTKDDVDAF 448
Rice_CpNifS                     ------------------HGVAIRSGHHCAQILHR-TLGINASARASLHFYNTKEEVDVF 360
E.coliSufS                      ------------------YGIAVRTGHHCAMPLMA-YYNVPAMCRASLAMYNTHEEVDRL 394
Pf_AcNifS                       PLWSNTFSSFDLVTFLDFKNICIRAGHHCASLLHKYYLKVPDTSRISIYFYNTPQEIKYL 528
                                                 :.  : :     :      :                .:. :

At_MtNifS                       YEMVKEGIDIKNIQWSQH--- 453
Rice_MtNifS                     YEMAKAGIDLKSIQWAQH--- 458
E.coli_IscS                     WEMYKQGVDLNSIEWAHH--- 404
Pf_MtNifS                       YEMELEKKNPSNDDIPKFIWT 553
At_CpNifS                       IVALADTVSFFNSFK------ 463
Rice_CpNifS                     VDALKDTIDFLTSEH------ 375
E.coliSufS                      VTGLQRIHRLLG--------- 406
Pf_AcNifS                       AQQIASTSFMLNEMKNEK--- 546
```

Figure 5. Amino acid alignment of mitchondrial (Mt) and chloroplastic (Cp) NifS proteins in Arabidopisis; also included is the NifS domain of cytosolic ABA3 in Arabidopsis. E.coli SufS and IscS are provided, as well as Typanosoma brucei (Tb) Mt and cytosolic (Cyt) NifS proteins. TbCytNifS does not show strong homology to any NifS proteins. The amino acid aligment score (top) and phylogram (bottom) are depicted.

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	TbCytNifS	451	2	TbMtNifS	437	30
1	TbCytNifS	451	3	AtCpNifS	463	11
1	TbCytNifS	451	4	AtMtNifS	453	29
1	TbCytNifS	451	5	ABA3	550	11
1	TbCytNifS	451	6	EColiIscS	404	34
1	TbCytNifS	451	7	EColiSufS	406	10
2	TbMtNifS	437	3	AtCpNifS	463	18
2	TbMtNifS	437	4	AtMtNifS	453	59
2	TbMtNifS	437	5	ABA3	550	8
2	TbMtNifS	437	6	EColiIscS	404	56
2	TbMtNifS	437	7	EColiSufS	406	19
3	AtCpNifS	463	4	AtMtNifS	453	18
3	AtCpNifS	463	5	ABA3	550	13
3	AtCpNifS	463	6	EColiIscS	404	22
3	AtCpNifS	463	7	EColiSufS	406	47
4	AtMtNifS	453	5	ABA3	550	11
4	AtMtNifS	453	6	EColiIscS	404	55
4	AtMtNifS	453	7	EColiSufS	406	19
5	ABA3	550	6	EColiIscS	404	16
5	ABA3	550	7	EColiSufS	406	16
6	EColiIscS	404	7	EColiSufS	406	19

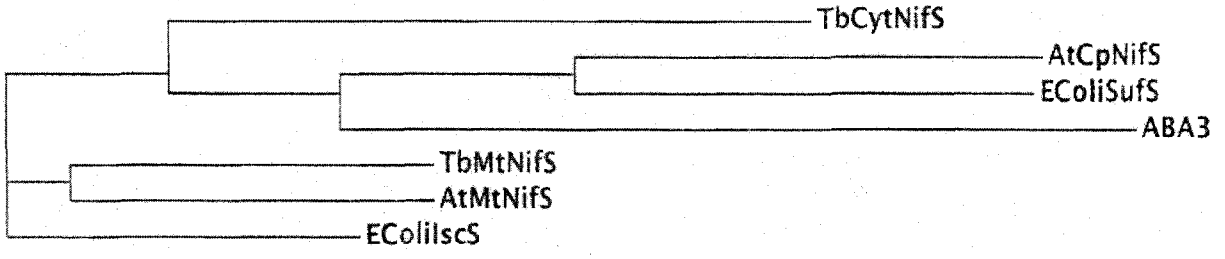
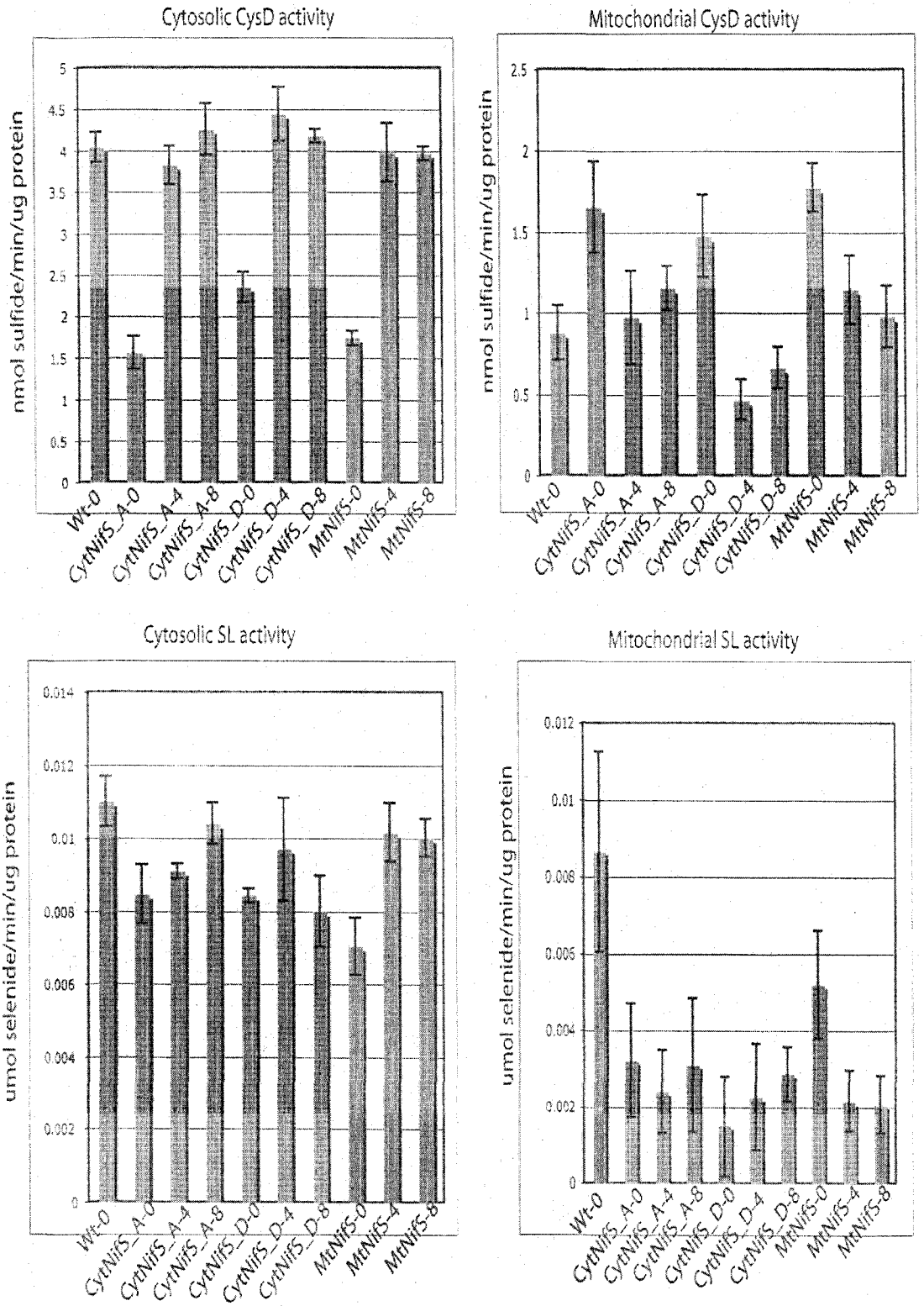


Figure 6. Measurement of cysteine desulfurase (CysD) and selenocysteine lyase(SL) activity in *Typanosoma brucei* cell lines, in which mitochondrial (Mt) and cytosolic (Cyt) NifS proteins were silenced. Cytosolic and mitochondrial protein fractions were collected from the different cell lines. The numbers represent how many days (0, 4, or 8) the cells lines were induced with the RNAi construct. Wt, wildtype; A and D, different lines of CytNifS RNAi. n= 4 replicates.



Acknowledgements

Gone are the days when a solitary scientist would work independently on the 14th floor of a state university, attempting to solve an esoteric scientific question. Thus, in addition to the hundred or so references I cite in this dissertation, I need to acknowledge that the science performed during my graduate studies is a collaboration among scientists who are energetic to share equipment and expertise and communicate their ideas.

Chapter 2

I would like to thank Lianne Pilon-Smits and Rien Pilon for their helpful comments and editing during the publication of this introductory chapter.

Chapter 3

I need to acknowledge several people for the research performed in this section. Gulnara Garifullina helped create the CpNifS overexpressing Arabidopsis plants. Ashley Ackley propagated and helped keep lines p38 and p55 alive while I was in Japan during the summer of 2004; she also assisted in the measurement of root lengths of plants grown on vertical plates (Figure 3). Salah Abdel-Ghany and I isolated chloroplasts from Arabidopsis plants (Figure 2 B,C). Although I assisted and was present and during the XANES and XAS analysis, Sirine Fakra and Matt Marcus were nearly single-handedly responsible for generating Figure 6. The microarray experiments could not have been performed without the facilities in the lab of Hideki Takahashi and the guidance and help of Keiki Ishiyama and Eri Inoue (Tables I and II).

Chapter 4

The continuation of the more elaborate microarray experiments in Chapter 3 would not have been possible without the enthusiastic support of Lianne Pilon-Smits, and once again the facilities and financial support from the lab of Hideki Takahashi. In particular, I need to acknowelge Eri Inoue who ran the microarray experiments. I owe a huge thanks to Ann Hess who performed the statistics of the microarray data sets in BioConductor-R, and who was able to explain complex statistical terminology in a

simplistic manner that I was able to understand. Ann also helped create Table S2. I also thank Nori Tamaoki who provided the mutant seeds in Figure 3, measured glutathione and ascorbic acid in Figure 4, and performed the macroarray experiments in Table S3.

Chapter 5

Among other things, I thank Lianne Pilon-Smits for helping me design and order primers for the CpNifS RNAi construct during my second full day in the lab in August, 2003. Her mentorship and guidance continued through the completion of the dissertation. The "holy grail" of the project- to show that CpNifS is essential for FeS proteins- was visualized by Rien Pilon; he co-advised Chapter 5, and his involvement and guidance was paramount to the project's success, particularly in helping me understand and measure electron transport in Photosystem II and I. I'm indebted to Salah Abdel-Ghany who cloned the RNAi construct from the constitutive promoter that I made into the inducible promoter and transformed Arabidopsis plants. He kept the project going while I was writing my dissertation for an MS degree in the Fall of 2004. He also helped me extract mRNA for cDNA synthesis used in Figure 1C. I thank Chris Cohu for performing Northern Blots (Figure S6) and for helping me set up the Qubit System for measuring carbon assimilation. We also both learned from each other about chlorophyll fluorometers. I thank Stephen Herbert for sharing his facilities and equipment for measuring PSI activity and oxygen consumption (Figure 4). He also helped design the experiments and assisted with the data acquisition. I also appreciate the time Paul Kugrens devoted to sharing his expertise with the transmission electron microscope. He helped me prepare samples, and took the photos seen in Figure 2.

In additions to the names I mention above, I also thank all the members of the Pilon/Pilon-Smits lab. I also appreciate the time dedicated by and the advice of my committee members, Cecil Stushnoff and Pat Bedinger, during my graduate studies.