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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.

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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

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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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

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

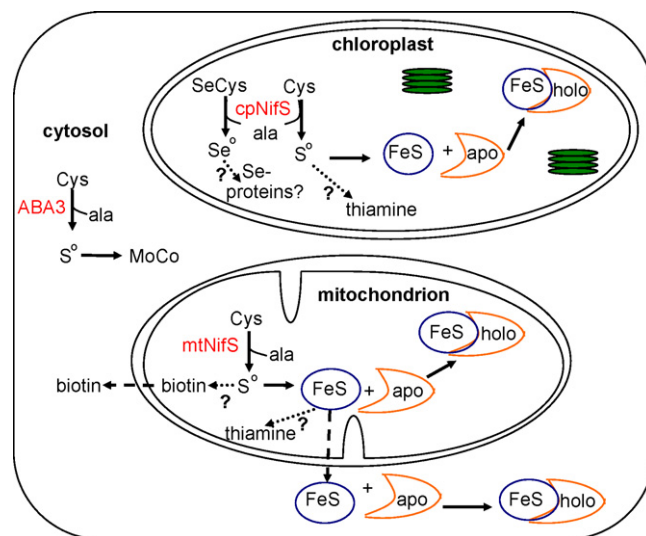


Fig. 1. Known (solid lines) and potential (dotted lines) functions of the three NifS-like proteins in plants (shown in red).

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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