CpSUF E ACTIVATES THE CYSTEINE DESULFURASE CpNIFS FOR CHLOROPLASTIC Fe-S CLUSTER FORMATION

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CpNifS, a cysteine desulfurase required to supply sulfur for iron-sulfur cluster biogenesis in Arabidopsis thaliana chloroplasts, belongs to a class of NifS-like enzymes with low endogenous cysteine desulfurase activity. Its bacterial homologue SufS is stimulated by SufE. Here we characterize the Arabidopsis chloroplast protein CpSufE, which has an N-terminal SufE-like domain and a C-terminal BolA-like domain unique to higher plants. CpSufE is targeted to the chloroplast stroma, indicated by GFP-localization and immunoblot experiments. Like CpNifS, CpSufE is expressed in all major tissues, with higher expression in green parts. Its expression is light-dependent and regulated at the mRNA level. The addition of purified recombinant CpSufE increased the V_max for the cysteine desulfurase activity of CpNifS over 40 fold, and decreased the K_M toward cysteine from 0.1 to 0.043 mM. In contrast, CpSufE addition decreased the affinity of CpNifS for selenocysteine, as indicated by an increase in the K_M from 2.9 to 4.17 mM, and decreased the V_max for selenocysteine lyase activity by 30%. CpSufE forms dynamic complexes with CpNifS, indicated by gelfiltration, native PAGE and affinity chromatography experiments. A mutant of CpSufE in which the single cysteine was changed to serine was not active in stimulating CpNifS, although it did compete with WT CpSufE. The iron-sulfur cluster reconstitution activity of the CpNifS-CpSufE complex toward apo-ferredoxin was 20-fold higher than that of CpNifS alone. We conclude that CpNifS and CpSufE together form a cysteine desulfurase required for iron-sulfur cluster formation in chloroplasts.

Iron-sulfur (Fe-S) cluster proteins perform a variety of biological roles in electron-transfer, catalysis, gene-regulation, and sensing of iron and oxygen (1). Iron-sulfur cluster proteins are particularly important to photosynthesis. Measurements of metal ions in Arabidopsis thaliana have indicated that about 70% of the Fe in green tissue is present in chloroplasts and 40% is found in the thylakoids (2). Estimates in other plants indicate that up to 90% of the Fe in leaves may be present in the chloroplasts (3). Within the thylakoids, the majority of the Fe is found in Fe-S cluster proteins that function in photosynthetic electron transport (4). Next to photosynthetic carbon fixation, other pivotal plastid functions that require Fe-S clusters include nitrogen assimilation, sulfur assimilation and pigment synthesis (for a review see: 5). Whereas all plastid types contain a number of important Fe-S cluster proteins, especially the green chloroplasts need to synthesize and maintain a variety of Fe-S proteins with at least 5 different cluster types (5).

In bacteria, three separate Fe-S formation machineries have been characterized (for a review see: 6). All systems include a NifS-like cysteine (Cys) desulfurase protein, which catalyzes the conversion of cysteine to alanine and elemental sulfur or the conversion of Selenocysteine (SeCys) to alanine and elemental Se. Every Fe-S machinery also has scaffold proteins thought to function in the pre-assembly of clusters before transfer to target proteins. The first discovered Fe-S assembly machinery was the Nif system of Azotobacter vinelandii, which is responsible for the formation of Fe-S clusters for nitrogenase (7). The second machinery was the Isc system first discovered in A. vinelandii and later in Escherichia coli, which has a housekeeping function in the formation of other cellular Fe-S proteins (8). Mitochondrial Fe-S assembly systems in eukaryotes are remarkably similar to this Isc system (6, 9). The third machinery was the suf system of E. coli and Erwinia chrysanthemi,
which appears to be responsible for the formation of Fe-S clusters under oxidative stress and iron limitation (10, 11, 12). Based on sequence similarities several of the plastid Fe-S biosynthesis components tentatively identified to date are most related to the bacterial suf cluster genes; other components are unique however (5).

Chloroplasts have their own Fe-S assembly systems (13, 14). Fe-S cluster assembly into radiolabeled freshly imported ferredoxin precursor was demonstrated using isolated intact chloroplasts (15). The reaction proceeds in the absence of cytosol (16). The presence of supersaturated amounts of oxygen in green tissues provides a challenge for the synthesis and maintenance of plastid Fe-S cluster proteins because of the sensitivity of these clusters to oxygen (1). Therefore, it can be anticipated that next to the synthesis of new clusters, chloroplasts must have unique mechanisms to replace or repair oxidatively-damaged clusters. Characterization of the chloroplastic Fe-S formation machinery started with the identification of a Cys desulfurase CpNifS (17, 18) and of scaffold proteins CpNfu2 (19, 20, 21) and CiplscA (22). CpNifS is the Cys desulfurase that converts cysteine into alanine and elemental sulfur for Fe-S formation. CpNfu2 can hold a transient Fe-S cluster. Insertion mutants in the CpNfu2 gene have a dwarf phenotype and are deficient in 2Fe-2S and 4Fe-4S proteins (20, 21). CiplscA is a putative alternative scaffold that can accept a 2Fe-2S cluster from CpNifS, which can be transferred to apo-ferredoxin (apoFD) in vitro (22). In addition, other Suf-type system components (23, 24, 25) and HCF101 (26) may assist the Fe-S formation in plastids. The CpSufBCD complex is an ATPase and may be involved in providing ferrous iron, or in transferring the Fe-S cluster from the scaffold protein to the target protein (23, 24, 25). HCF101 (high chlorophyll fluorescence 101) encodes a NifH-related P-loop ATPase that seems to be required for 4Fe-4S but not 2Fe-2S assembly in chloroplasts (26).

Since cysteine was identified as the sulfur source for Fe-S formation in chloroplasts (13, 14), the Cys desulfurase activity of CpNifS is likely essential for Fe-S formation in chloroplasts. Indeed, the depletion of CpNifS led to the loss of Fe-S reconstitution activity of chloroplast stroma (27). This Cys desulfurase of chloroplasts is distinct from the Cys desulfurases NifS and IscS of the Nif and Isc type assembly systems and is more similar in sequence to SufS (18). In vitro, the purified CpNifS has a much lower Cys desulfurase activity than SeCys lyase activity (18). However, the Fe-S cluster reconstitution activity of CpNifS in stroma is 50-80 fold higher than that of CpNifS alone, suggesting some factors - most likely proteins - are activating the Cys desulfurase activity of CpNifS (27). The activation mechanism has been unclear until the characterization of CpSufE in this study. CpSufE is the latest component of the Suf-type Fe-S formation system identified in Arabidopsis chloroplasts. We show here that CpSufE forms a complex with CpNifS, thus stimulating Cys desulfurase activity over 40-fold, and enhancing CpNifS-dependent Fe-S reconstitution in vitro.

MATERIALS AND METHODS

Cloning and plasmid construction - The A. thaliana CpSufE coding sequence was amplified by PCR using cDNA as a template. cDNA was prepared from DNase-treated total RNA prepared from two-week old seedlings as described (18). Primers used for CpSufE amplification were SuF-precursor and SuF-Bam (Table 1). The PCR product was digested with NcoI and BamHI and then ligated into vector pET11d (Novagen, Madison, WI), digested with the same restriction enzymes to produce plasmid pPrSufE. To subclone the mature sequence of CpSufE in pET28a (Novagen, Madison, WI) for expression as a His6-tagged protein, PCR was performed with a set of nested primers, SuF-mature and SuF-Bam (Table 1). Plasmid pPrSufE was used as a template. The PCR product was digested with NdeI and BamHI and subcloned in vector pET28a, which was digested with the same restriction enzymes to produce plasmid pMSufE.

To change the single cysteine in the mature sequence of CpSufE to serine, recombinant PCR was performed. In the first round, two fragments were amplified with primer set T7 and SuFEC55-R, and primer set T7 terminator and SuFEC55-F, respectively (Table 1). pMSufE was used as template. The two products from the first round of PCR were together used as template for the second round of PCR, with primers SuF-mature and SuF-Bam (Table 1). The resulting
PCR product was digested with *Nde*I and *BamH*I and subcloned in vector pET28a to produce plasmid pMSufEC65S.

For GFP localization, the transit peptide coding sequence of CpSufE was amplified with flanking primers *SufE-GFP-F* and *SufE-GFP-RT* (Table 1), while the full-length protein sequence was amplified with flanking primers *SufE-GFP-F* and *SufE-GFP-R* (Table 1). The pPrSufE plasmid was used as a template. PCR products were digested with *Sal*I and *Nco*I, and inserted into the *Sal*I/*Nco*I digested GFP reporter plasmid 35W-SGFP(S65T) (28) to create the plasmids TP/SufE-GFP and Full length/SufE-GFP, respectively.

For site-directed mutagenesis of cysteine388 in mature CpNifS to serine, a strategy was used similar to the one described above for the CpSufE mutant. In the first round of PCR, two fragments were amplified with primers T7 and *NifSC388S-R*, and primers T7 terminator and *NifSC388S-F* respectively (Table 1), using pMNFS-8 (18) as a template. The final PCR product was digested with *Nco*I and *BamH*I, and ligated into vector pET11d, resulting in pMNFSC388S. All constructs were verified by DNA sequencing. The plasmids used for protoplast transformation were prepared using the Plasmid Midi Kit (Qiagen, Valencia, CA).

**Sequence analysis and alignments** - Sequence analysis was performed using the Mac Vector sequence analysis software (International Biotechnologies, New Haven, CT). Searches for sequence similarity were performed using the BLAST network service provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Sequence alignment was performed using ClustalW at European Bioinformatics Institute, ExPASY Proteomics tools (http://www.ebi.ac.uk/clustalw).

**Preparation of proteins** - For overexpression of CpSufE, *E. coli* BL21 (DE3) codon+ (Stratagene, La Jolla, CA) was transformed with plasmid pMSufE or with pMSufEC65S for the mutant protein. Two liter of LB medium containing 50 µg ml⁻¹ kanamycin was inoculated with 1/100 volume of overnight culture. Cells were grown at 37 °C to an OD₆₀₀ of 0.5, and expression was induced by addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) followed by incubation for 3 h at 37°C. The culture was chilled on ice, and the cells were collected by centrifugation for 5 min at 5,000 g at 4°C. From here on, all procedures were performed at 4 °C except where mentioned. The bacterial pellet was washed with 150 mM NaCl and resuspended in 50 mM Tris-HCl, pH 7.5, then passed twice through a French press (8,000 p.s.i) to disrupt the cells. The lysate was centrifuged for 20 min at 12,500 x g and the cleared supernatant was loaded at a flow rate of 3 ml min⁻¹ onto His-Bind iminodiacetic acid (IDA) agarose (Novagen, Madison, WI) in a 1.6 x 20 cm column, which was saturated with NiSO₄, washed and equilibrated in 50 mM Tris-HCl, pH 7.5. The column was washed with four volumes of 50 mM Tris-HCl, pH 7.5; followed by 4 volumes of 1 M NaCl, 50 mM Tris-HCl pH 7.5; 6 volumes of 50 mM Tris-HCl, pH 7.5 again and two volumes of 0.1 M imidazole in 50 mM Tris-HCl, pH 7.5, respectively. His-tagged CpSufE was eluted with 4 volumes of 1 M imidazole in 50 mM Tris-HCl, pH 7.5. Fractions of 6 ml were collected. Peak fractions were pooled and dialyzed overnight against 25 mM Tris-HCl, pH 7.5. Pure His-tagged CpSufE ran as a single band on SDS-PAGE after staining with Coomassie Brilliant Blue.

To produce cleaved CpSufE, the pooled peak fractions were dialyzed overnight against 20 mM Tris/HCl, pH 8.4, followed by incubation with thrombin in a 1:1000 w/w ratio (thrombin : target protein) at 4 °C for 8 h in 20 mM Tris/HCl, pH 8.4, 150 mM NaCl, 2.5 mM CaCl₂ as suggested by the manufacturer (Novagen, Madison, WI). The cleavage mixture was subsequently applied to a 10 x 1 cm Resource-Q column, (Amersham Biosciences, Piscataway, NJ) equilibrated in 25 mM Tris/HCl, pH 8.0, at room temperature and connected to a Summit HPLC system (Dionex, Sunnyvale, CA). The column was eluted with a linear gradient from 0 to 1 M NaCl in 25 mM Tris-HCl, pH 8.0, and fractions of 2 ml were collected. Elution was monitored by detection of the OD at 280 nm and 220 nm. The purified cleaved CpSufE was dialyzed overnight against 25 mM Tris/HCl, pH 7.5, and stored frozen at -80 °C before use in activity assays. Typical yields were 5-10 mg l⁻¹ of culture. The purified protein migrated as a single band on SDS-PAGE (12.5% gel) and ran as a single peak in
analytical HPLC runs on a 1 ml Resource Q column (Amersham Biosciences, Piscataway, NJ). The His6-tagged and cleaved CpSufE<sub>C65S</sub> were purified essentially as the WT CpSufE protein. WT CpNifS protein was prepared as described before (18). For overexpression of CpNifS<sub>C388S</sub>, <i>E. coli</i> BL21 (DE3) codon<sup>+</sup> (Stratagene, La Jolla, CA) was transformed with plasmid pMNFS<sub>C388S</sub>. The recombinant CpNifS<sub>C388S</sub> was prepared essentially as WT CpNifS (18). Purified CpNifS<sub>C388S</sub> was eluted from a calibrated 1 x 30 cm Superdex-S200 gel-filtration column (Amersham Biosciences, Piscataway, NJ) at the same retention time as the WT protein, and a native molecular mass of 83 kDa was calculated, suggesting that CpNifS<sub>C388S</sub> is a dimer like the wild type protein. Holo- and apo-Fd were prepared as described (27).

**Enzyme assays** - Cys desulfurase activity was assayed at 25°C essentially as described (29). Briefly, 160 µl reaction mixture contained 25 mM Tris-HCl pH 7.4, 100 mM NaCl, 2.5 µM enzyme (0.11 mg/ml CpNifS, 0.09 mg/ml CpSufE), 10 µM pyridoxal 5' phosphate (PLP, Acros Organics, Morris Plains, NJ), 1 mM DTT (Roche, Mannheim, Germany), and 500 µM cysteine (Sigma, St. Louis, MO). The reaction was stopped by addition of 20 µl 20 mM N,N dimethyl-p-phenylenediamine in 7.2 M HCl. Methylene blue was formed by addition of 20 µl 30 mM FeCl<sub>3</sub> in 1.2 M HCl and was assayed by measuring the absorbance at 670 nm. The SeCys lyase activity was measured as described (18). One unit of enzyme activity corresponds to 1 micromole substrate converted min<sup>-1</sup>. To estimate kinetic constants for both the Cys desulfurase and SeCys lyase activities of CpNifS the reaction velocities were measured over a wide range of substrate concentrations (0.01 – 20 mM). The data in Michaelis-Menten plots were fitted by an iterative method to estimate K<sub>M</sub> and V<sub>max</sub> values, using the software program Enzfitter (Biosoft, Cambridge, UK). The Fe-S reconstitution assay was performed as described before (27). For all statistical analyses, the JMP-IN software (SAS institute, Cary, NC) was used.

**Gelfiltration** – Sizes of protein complexes were estimated by gelfiltration experiments, as described before (27). The column used was a 1x30 cm Superdex-200 column (Pharmacia, Piscataway, NJ), which was connected to a summit HPLC system with a UVD170 detector and controlled by Chromleol software (Dionex, Sunnyvale, CA). The loop size was 0.2 ml. The column was equilibrated in 25 mM Tricine/KOH, pH 7.9, 50 mM KCl. The flow rate was 0.75 ml min<sup>-1</sup> and fractions were collected every 0.5 min. Elution was monitored by absorbance at both 280 and 220 nm and by immunoblotting of collected fractions. The void volume was determined with blue dextran. Standards used for calibration were IgY, BSA, ovalbumin, chymotrypsinogen and RNAse.

**Protein coelution experiments** – 100 µg His-tagged CpSufE (WT or mutant) and possible partner proteins were mixed at room temperature in 500 µl of buffer (50 mM Tris-HCl, pH 7.5), and loaded on a 0.5 ml His-Bind iminodiacetic acid (IDA) agarose column (Novagen, Madison, WI). The column was washed with 2 ml of buffer, and subsequently with 2 ml of 1 M NaCl, 50 mM Tris-HCl pH 7.5, followed by 2 ml of 50 mM Tris-HCl, pH 7.5 again, and 1 ml of 50 mM Tris-HCl pH 7.5, 0.1 M imidazole. Finally, the column was eluted with 2 ml of 1 M imidazole, 50 mM Tris-HCl pH 7.5, and one ml fractions were collected. Samples incubated with cysteine were eluted with all solutions containing 1 mM cysteine, while samples incubated without cysteine were eluted with solutions free of cysteine. Proteins eluted with 1 M imidazole were analyzed by SDS-PAGE and staining with Coomassie Brilliant Blue.

**Separation of CpNifS-CpSufE complexes on native gel** – 5 µg of CpSuF (WT or mutant) was mixed with 5 µg of CpNifS (WT or mutant) in a final volume of 15 µl, with or without 1 mM cysteine. After incubation at room temperature for 10 minutes, proteins were mixed with an equal volume of 20% w/v glycerol, 250 mM Tris/HCl pH 6.8, and separated by native PAGE, using a 4% stacking gel, a 10% separating gel, and the Laemmli buffer system with the omission of SDS. 5 µg of each single protein was loaded as control. The gel was stained with Coomassie Brilliant Blue.

**Plant sampling** – Arabidopsis thaliana (Ecotype Columbia-0) plants were grown on soil with supplementary light on a 15-h-light/9-h-dark cycle for 4 weeks. Total leaf homogenate, chloroplast stroma, and RNA from different tissues were prepared as described (18). For light
regulation analysis of CpSufE, Arabidopsis plants were grown on half-strength Murashige and Skoog (MS) agar medium (30) for 2 weeks, either on a 15-h-light/9-h-dark cycle or in complete darkness. Protein and RNA preparations from total leaf homogenate were described before (18).

Sub-cellular localization of GFP-fusion proteins - The plasmids TP/SufE-GFP or Full length/SufE-GFP were transformed into Arabidopsis protoplasts and expressed proteins were observed under a confocal microscope, as described (22).

Antibodies and immunoblotting – Cleaved CpSufE in 100 mM NaCl, 25 mM sodium phosphate, pH 7.5 was used to raise polyclonal antibody in rabbits at a commercial facility (PRF&L, Canadensis, PA). The CpNifS antibodies have been described (18). The Hsp70 antibody was purchased from Sigma (St. Louis, MO). RuBisCo antibody was purchased from AgriSera (Vannas, Sweden). Immunoblotting was performed as described (18). Band intensities were quantified with ImageJ software (NIH, Bethesda, MD).

RNA blot analysis – Total RNA from different Arabidopsis tissues was prepared, electrophoresed, and probed with a 32P-labeled 900 bp CpSufE cDNA, essentially as described before (18). Band intensities were quantified with ImageJ software (NIH, Bethesda, MD).

RESULTS

Identification of CpSufE and sequence characteristics – CpNifS is a Cys desulfurase (17, 18) required for iron sulfur cluster formation in the chloroplast (27). However, compared to most Cys desulfurases with a housekeeping role in Fe-S cluster formation, the Cys desulfurase activity of purified CpNifS is very low, despite a high selenocysteine lyase activity (18). When the in vitro reconstitution activity of purified CpNifS protein was compared to the activity of CpNifS in stromal extracts it was concluded that CpNifS in stroma is about 50-80 times more active (27). Thus a stimulatory activity must be present in plastids. We considered that this stimulatory activity could at least in part be explained if plastids contain a homologue of SufE, a protein which stimulates the Cys desulfurase activity of the bacterial SufS proteins. A SufE-like sequence (At4g26500) was identified by a BLAST search within the Arabidopsis genome database (TAIR; http://www.arabidopsis.org), using the E. coli SufE (29) as a query sequence. The genomic sequence of At4g26500 contains a single predicted exon. A cDNA containing the full coding sequence was obtained by RT-PCR. Sequence analysis of the cDNA confirmed the presence of a single uninterrupted coding sequence in the genome (not shown). The open reading frame is predicted to encode a full-length precursor protein of 371 amino acids (see Fig. 1A), including a putative chloroplast targeting peptide (66 amino acids) as predicted by the TargetP program (www.expasy.org, and see: 31). Because of the predicted location and similarity with SufE proteins we named At4g26500 CpSufE, for chloroplastic SufE. The predicted mature polypeptide is 305 amino acids long, with a molecular mass of 33.6 kDa and an isoelectric point of 4.87. The mature size of the Arabidopsis CpSufE homologue is much larger than what was reported for the bacterial SufE proteins, which are about 15 kDa in size (29, 32).

A sequence alignment was performed to determine the similarity of the putative Arabidopsis CpSufE with possible SufE homologues from various organisms, as shown in Figure 1. The predicted cleavable chloroplast targeting sequence of the Arabidopsis protein is underlined. A similar N-terminal extension, predicted to be a transit sequence by the TargetP program, is not found in the prokaryotic homologues, as expected. All SufE homologues show sequence conservation in the mature protein domain, including a highly conserved single cysteine that was shown to be critical for function in the bacterial proteins (29, 33). This SufE domain region corresponds to the N-terminal half of the predicted mature proteins in Arabidopsis and rice. In this region the plant proteins are most related in sequence to the cyanobacterial SufE. For example, the sequence motif GCVSQV, which includes the conserved cys, is conserved between Arabidopsis CpSufE, rice and cyanobacterial SufE but different in the other bacterial proteins. The two plant proteins are predicted to have a C-terminal domain of about 150 amino acids, which is absent from the prokaryotic homologues. The last 88 residues of this plant-specific C-terminal
protein domain show very good sequence similarity to *E. coli* BolA (45% identity and 61% similarity). BolA of *E. coli* may function in the control of cell shape in response to nutrition (34). BolA-like proteins are found ubiquitously but so far a molecular function has not been identified for these proteins experimentally (34). However, recent bioinformatic approaches have suggested that BolA domains may interact with members of the monothiol glutaredoxins (35). The rice and Arabidopsis proteins display good sequence conservation over their entire length (42% identity), but the conservation in the BolA-like domain is even higher, with 72% identity; this sequence conservation suggests a significant function for the BolA region of CpSufE in vivo.

**Localization of CpSufE in chloroplasts** - The TargetP program predicted a cleavable transit sequence of 66 amino acids and a chloroplast localization of CpSufE. To examine the subcellular localization, we constructed fusions with the green fluorescent protein (GFP). In the first construct, the CpSufE transit peptide was fused to the N-terminus of GFP; in a second construct the full-length precursor CpSufE was fused to the N-terminus of GFP. GFP alone expressed from the same constitutive promoter was used as a control. Constructs were separately introduced into Arabidopsis protoplasts and the localization in cells was analyzed using confocal laser microscopy. Fluorescence corresponding to GFP expressed without a transit sequence was excluded from the chloroplasts as expected (Fig. 2A, upper panel). In contrast, green fluorescence from the transit sequence fusion was localized to the chloroplast stroma, as indicated by the overlay of green fluorescence and red auto-fluorescence (Fig. 2A, middle panel). Green fluorescence from the full-length CpSufE coupled to GFP was localized to discrete locations in the chloroplasts (Fig. 2A, lower panel).

The localization of CpSufE in chloroplast stroma was verified by immunoblotting (Fig. 2B). CpSufE antibody detected as little as 0.5 ng purified CpSufE. The antibody detected a protein of 37 kDa in total leaf homogenate (TH) and this signal was quantitatively recovered in the chloroplast stroma fraction (St), (Fig 2B). The pre-immune serum at the same dilution did not recognize any bands in these samples (data not shown). These data indicate the presence of CpSufE in stroma. Based on the band intensities, we estimate that the amount of CpSufE present in 20 µg of stromal protein is between 10 and 20 ng. Thus the abundance of CpSufE in stroma is between 0.05 and 0.1% of total protein, similar to the abundance (0.06%) reported for CpNifS (27). In conclusion, the immunoblotting and GFP experiments strongly suggest a stromal localization of CpSufE.

**Expression analysis of CpSufE** - Expression patterns can give clues about the function of a protein. To determine the CpSufE mRNA and protein expression patterns in different Arabidopsis tissues we performed RNA blot and immunoblot analyses (Fig. 3) and quantified band intensities. The RNA blot analysis revealed that CpSufE is expressed in all tested tissues with a 1.7 fold higher expression level in leaves compared to non-green tissues (roots and flowers). The expression of the CpSufE transcript in the stem was 1.4-fold higher than in the roots and flowers (Fig. 3B). Immunoblots confirmed this expression pattern at the protein level and showed that CpSufE and CpNifS have comparable expression patterns. As quantified, the CpSufE protein expression in leaf was 1.3-fold higher than in the stem, flower and root (Fig. 3A). The presence of CpSufE in plastids and the elevated expression in green tissues are typical for proteins involved in photosynthesis. Many photosynthesis-related genes are regulated by light. We therefore investigated if CpSufE expression is influenced by light. CpSufE protein was found to be expressed less by 2.5-fold in plants grown in the dark, compared to light-grown plants (Fig. 3C) and this can be ascribed to 2.3-fold lower expression of the CpSufE mRNA in the dark (Fig. 3D). However, even though there was lower expression of CpSufE in the dark there was still significant expression, and the effect of light on the expression of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, a known light-induced chloroplast protein was larger in comparison.

CpSufE stimulates Cys desulfurase activity and changes the substrate affinity of CpNifS - To allow us to investigate the activity of CpSufE, we purified the mature-sized protein. The N-terminus of the protein was fused to a six-histidine tag and a thrombin cleavage site (Fig. 4A) for efficient purification by immobilized metal ion affinity
chromatography (Fig. 4B). To remove the His6 tag, the protein was incubated with thrombin at 4 °C until the His6 tag was completely cleaved as judged by SDS-PAGE followed by staining for total protein with Coomassie Brilliant Blue, and CpSufE was purified from the mixture by HPLC using a Resource-Q anion exchange column (Fig. 4B). The purified CpSufE was a colorless protein with a mass of about 40 kDa as judged by SDS-PAGE followed by staining for total protein with Coomassie Brilliant Blue, and CpSufE was purified from the mixture by HPLC using a Resource-Q anion exchange column (Fig. 4B). The purified CpSufE was a colorless protein with a mass of about 40 kDa as judged by SDS-PAGE followed by staining for total protein with Coomassie Brilliant Blue, and CpSufE was purified from the mixture by HPLC using a Resource-Q anion exchange column (Fig. 4B).

CpSufE alone did not show any activity in either the Cys desulfurase assay (Fig. 5C) or the SeCys lyase assay (Fig. 5B), both of which are activities displayed by CpNifS. To test if CpSufE changes the kinetic properties of CpNifS, the activities of CpNifS alone and of CpNifS plus CpSufE in a 1:1 molar ratio were assayed over a concentration range for both substrates (Table II). CpNifS alone displayed a barely detectable level of Cys desulfurase activity ($V_{\text{max}} = 0.0013 \text{ mol/min/mg}$) but displayed high SeCys lyase activity ($V_{\text{max}} = 2.44 \text{ mol/min/mg}$), in agreement with our previous results (18). However when CpSufE was added to CpNifS in a 1:1 molar ratio, we observed a 40-fold increase in the $V_{\text{max}}$ for Cys desulfurase activity compared to CpNifS alone. In contrast, the $V_{\text{max}}$ for SeCys lyase activity was reduced by 30%. The addition of CpSufE to CpNifS caused the $K_M$ for cysteine to decrease 2.5-fold. On the other hand, the $K_M$ for selenocysteine increased slightly (1.4-fold). Thus, the $V_{\text{max}}/K_M$ ratio of CpNifS was increased 100-fold by CpSufE. Titration experiments at saturating substrate concentration in which the amount of CpNifS was kept constant while CpSufE was varied indicated that the Cys desulfurase activity of CpNifS depended on the amount of CpSufE present. The stimulation by CpSufE reached an apparent saturation point at a CpSufE: CpNifS molar ratio of 5:1, at which point we observed a 60-fold stimulation (Fig. 5A).

CpNifS has 5 cysteines, one of which, Cys388, is conserved and predicted to be required for the Cys desulfurase activity (18) based on similarity with bacterial CsdB (36) for which a structure is published (37). To investigate if this cysteine in CpNifS is required for the activity of the protein we purified a mutant CpNifSC388S in which the cysteine was altered to serine. Purified CpNifSC388S displayed the same absorbance spectrum and elution profile from a gel filtration column as the WT protein. Similar to what had been found for the CsdB Cys-mutant (36), the mutant CpNifSC388S protein retained about 80% of its SeCys lyase activity (Fig. 5B). This indicates that under the assay conditions the decomposition of selenocysteine does not depend on the active site cysteine and that the mutant CpNifSC388S enzyme was folded and active. However, the CpNifSC388S mutant protein had no detectable Cys desulfurase activity anymore, neither alone nor with CpSufE present. Thus the conserved cysteineC388 is essential for Cys desulfurase activity of CpNifS, but not for SeCys lyase activity.

CpSufE has a single conserved cysteine, Cys65, in its mature sequence. To test the requirement of the thiol group for CpSufE activity we expressed and purified a mutant in which the cysteine was changed to serine. Like the wild-type CpSufE, the mutant CpSufEC65S did not show activity by itself (Fig. 5B, C). Only WT CpSufE but not CpSufEC65S displayed stimulation of CpNifS Cys desulfurase activity, indicating that the conserved cysteineC65 is essential for this function (Fig. 5C). Both CpSufE and CpSufEC65S had a similar small but noticeable negative effect on the SeCys lyase activity of CpNifS.

The Cys65Ser mutation in CpSufE is dominant negative in vitro – We tested the effect of the presence of the CpSufE and CpNifS cyst mutants on the activity of WT CpNifS and WT CpSufE. Interestingly, the presence of CpSufEC65S severely inhibited the stimulatory activation of CpNifS by WT CpSufE in a concentration dependent manner, thus showing a dominant negative effect in vitro. In marked contrast, even a five-fold excess of mutant CpNifSC388S did not inhibit the activation of WT CpNifS by CpSufE (Fig. 6). A straightforward interpretation of these observations is that in these incubations, the
mutant CpSufE<sub>C65S</sub> titrates WT CpNifS into an inactive complex, while mutant CpNifS<sub>C388S</sub> does not titrate away WT CpSufE from WT CpNifS. The difference may be explained by differences in affinity between mutant and WT proteins that in turn may be related to the ability to decompose cysteine or accept S.

**CpSufE complexes with CpNifS** – The observed alterations of the catalytic properties of CpNifS by CpSufE prompted us to investigate if CpSufE and CpNifS form a complex. To address this question, we first performed a gel filtration analysis (Fig. 7). The native molecular weight of recombinant CpSufE and of the CpSufE<sub>C65S</sub> mutant was determined by comparing the elution time from a Superdex 200 gel filtration column with standards. In gel filtration experiments, CpSufE eluted in single peak at 18.5 min (Fig. 7A, trace 1). The same elution profile was seen for CpSufE<sub>C65S</sub> (not shown). The retention time of 18.5 min corresponds to an estimated molecular mass of ~70 kDa, indicating that purified recombinant CpSufE and the CpSufE<sub>C65S</sub> mutant were homodimeric proteins. CpNifS eluted as a single peak at 17.6 min (Fig. 7A, trace 2), which corresponds to a size of 86 kDa, in agreement with the mass expected for homodimeric CpNifS (18). When WT CpSufE and CpNifS (WT) were mixed in a 1:1 molar ratio, and applied to the gel filtration column, we found most of the protein to elute in a peak with a retention time of 17.2 min (Fig. 7A, trace 3). Comparison to standards indicated an apparent molecular mass of ~110 kDa, indicating the formation of a complex that is significantly smaller than the added mass of two dimers together. The published structure for the homologous *E. coli* SufS protein suggests a very stable dimer structure for CpNifS (37). Indeed the CpNifS dimer is very stable and we have never observed monomers of this protein. We thus consider it likely that trace 3 in Fig 7A represents a hetero-trimer of two CpNifS subunits and one CpSufE subunit, which is in agreement with the estimated size of the complex. Indeed both CpSufE and CpNifS were found to be present in the peak as judged from SDS-PAGE, but CpSufE seemed less abundant in the peak fractions (Fig. 7B, gel 3), and addition of more CpSufE shifted the complex to a higher molecular weight (not shown). When the mutated CpSufE<sub>C65S</sub> was incubated with CpNifS and loaded onto the gel filtration column a peak with a retention time of 16.5 min was observed (Fig. 7A, trace 4), corresponding with a complex with an apparent size of ~150 kDa, which is in good agreement with the cumulative molecular mass of a CpSufE dimer and a CpNifS dimer, a composition that is confirmed by SDS-PAGE analysis. These data indicate that the mutant CpSufE retains CpNifS-binding ability, and may have a higher affinity for CpNifS than the WT CpSufE. When the cysteine mutant of CpNifS was substituted for the WT protein and incubated with CpSufE, the same elution pattern was obtained that was observed with WT CpNifS (data not shown). Therefore, the affinity of WT and mutant CpNifS for CpSufE appears to be similar.

As mentioned above, the WT CpSufE and CpNifS proteins appear to form a NifS<sub>2</sub>-SufE trimer when incubated at lower concentrations, but this complex appears to shift to a heterotetramer when CpSufE concentration is increased. A similar tetrameric complex was observed when the concentrations of both proteins were increased ten-fold (Fig. 7C). The formation of the putative trimeric and tetrameric complexes and the effect of cysteine on complex formation was further investigated by native PAGE (Fig. 7D). CpNifS and CpSufE are both negatively charged and have a very similar charge/mass ratio. Therefore, we can expect that the mobility in native PAGE will be mainly determined by the size of the native protein complex. When individual proteins were loaded onto the native gel, CpNifS migrated as a single band, presumably its dimeric form; WT CpSufE also migrated as a single band, presumably as a dimer. The mutant CpSufE<sub>C65S</sub> also mostly migrated as this presumed dimeric form, but a small fraction migrated as a band with higher mobility, presumably the monomeric form. When the WT CpSufE and WT CpNifS were incubated in the absence of their substrate cysteine, the dimeric CpNifS disappeared, as did most of the dimeric CpSufE, and instead a smear appeared representing a larger complex of CpNifS and CpSufE, possibly a heterodimer that is perhaps somewhat unstable under the experimental conditions (Fig. 7D). In the presence of cysteine the same protein mixture formed two more distinct complexes (Fig. 7D, band II). We hypothesize that these two newly appearing bands, labeled I and II, are a trimeric NifS<sub>2</sub>-SufE complex.
and a NifS₂-SufE₂ heterotetramer, respectively. The desulfuration triggered by addition of the cysteine substrate appears to stimulate the formation of the putative tetramer, which may be the most active catalytic form of the CpNifS-CpSufE cysteine desulfurase complex. The latter is supported by the finding that an increase in CpSufE:CpNifS ratio shifts the complex to a higher molecular weight (judged from gelfiltration experiments) and at the same time increases Cys desulfurase activity of the complex (Fig. 5A).

When the WT CpSufE protein was incubated with the mutant CpNifS₃₈₈₅ in the absence of cysteine, the results were comparable to incubation of both WT proteins. There was no effect, however, of the addition of cysteine, likely because no desulfuration could be carried out by the mutant CpNifS₃₈₈₅. When the mutant CpSufE₆₆₅₅ was incubated with WT CpNifS only the putative heterotetramer (band II) was observed, regardless of the presence of cysteine, and neither dimeric CpNifS nor dimeric CpSufE were observed (Fig. 7D). A straightforward interpretation is that the mutant CpSufE₆₆₅₅ has a stronger affinity for CpNifS, or that the CpSufE₆₆₅₅-CpNifS complex is more stable. Finally, incubation of mutant CpNifS₃₈₈₅ with mutant CpSufE₆₆₅₅ resulted in only the putative trimeric complex (band I), regardless of the presence of cysteine. These results are similar to those observed when the mutant CpNifS₃₈₈₅ was incubated with WT CpSufE, and may indicate that mutant CpNifS₃₈₈₅ has a reduced binding ability to CpSufE. These native PAGE results confirm the presence of different CpSufE-CpNifS complexes and also explain the dominant negative effect of CpSufE₆₆₅₅ when added to WT. The NifS and SufE proteins form complexes even when the active site Cys residues in both proteins are changed to Serine. Still, the active site residues in CpNifS and CpSufE clearly affect the nature of the complexes that are formed. Furthermore the effect of Cys on complex formation between the WT proteins suggest that conformational changes occur in CpNifS upon the completion of a round of desulfuration, which in turn may facilitate a release of CpSufE from the complex. It thus appears that CpSufE is in dynamic exchange between a “free” CpSufE and several complexed states.

Investigating the formation of the CpSufE-CpNifS complex – The formation of a CpSufE-CpNifS complex was further investigated in Ni-column coelution experiments. We used the His₉-tagged CpSufE and incubated the protein with an equal amount of CpNifS before loading onto a Ni-IDA column. The column was treated with a series of salt washes before elution with 1 M imidazole. Samples of the loaded and imidazole-eluted proteins were analyzed by SDS-PAGE (Fig. 8A). Wild-type CpNifS was found to co-elute with the His-tagged wild-type CpSufE. Control experiments showed that CpNifS did not bind to the nickel column by itself (data not shown). The same elution pattern was found for CpNifS and His-tagged CpSufE regardless of whether the proteins had the wild-type sequences or were mutated at the essential cysteine residues. Therefore in this Ni-column binding assay, CpNifS and CpSufE are again shown to interact and this interaction does not require the conserved Cys residues in either protein, which are however needed for Cys desulfurase activity. Another chloroplast protein, CplscA, which may serve as a chloroplast scaffold protein for Fe-S assembly (22) did not co-elute with CpSufE or with CpSufE and CpNifS (data not shown).

The observation of the putative trimeric NifS₂-SufE complex in addition to the putative NifS₂-SufE₂ heterotetramer suggests the involvement of the CpSufE monomer in the formation of the complex. This hypothesis was further investigated by Ni-column coelution experiments. We mixed dimeric His-tagged CpSufE with separately purified dimeric untagged (cleaved) CpSufE and CpNifS, either with or without cysteine. The protein mixture was loaded onto a Ni-IDA column and eluted with 1 M imidazole to select for His-tagged CpSufE and any proteins associated with it. The cleaved untagged CpSufE, which does not bind to the column by itself, and CpNifS were found to co-elute with His-tagged CpSufE (Fig. 8B, imidazole eluates I). This presence of untagged CpSufE in a complex with tagged CpSufE can be explained by the formation of a heterotetramer containing a cleaved CpSufE, a His-tagged CpSufE and two CpNifS subunits (Fig. 8C). Cysteine had no notable effect in this experimental protocol. When the mutant CpSufE₆₆₅₅ was mixed with the His-tagged CpSufE and CpNifS, the cleaved CpSufE₆₆₅₅ also
coeluted with the other two components in the 1 M imidazole fraction (Fig. 8B, imidazole eluates II). However, much less complex coeluted with the His-tagged WT CpSufE when it had to compete with the CpSufEC65S mutant, again indicating that the mutant CpSufEC65S has a higher affinity for CpNiFS than WT CpSufE, and explaining the dominant negative effect of the mutant CpSufEC65S on stimulation of CpNiFS Cys desulfurase activity by WT CpSufE. The formation of the CpSufE-CpSufEtag-CpNiFS complex suggests that CpSufE is incorporated into the CpSufE-CpNiFS complex in the form of monomers, as schematically depicted in figure 8D.

*CpSufE enhances the Fe-S reconstitution in ferredoxin 20-fold* – To quantitatively measure Fe-S cluster formation, a Fe-S reconstitution assay for ferredoxin was used (27). The effect of CpSufE on the Fe-S cluster formation in ferredoxin was tested in these CpNiFS-dependent reconstitution assays (Fig. 9). CpSufE by itself did not mediate any Fe-S cluster formation, while CpNiFS only displayed a very low level of activity. However, the mixture of 5 μg (or 0.8 μM) CpNiFS and 4 μg (or 0.8 μM) CpSufE reconstituted ~21 μg (or 12 μM) ferredoxin, equivalent to the activity of 100 μg (or 16 μM) pure CpNiFS. This indicated a 20-fold enhancement of CpNiFS-dependent Fe-S cluster formation by CpSufE.

**DISCUSSION**

The Cys desulfurase activity of CpNiFS is required for Fe-S formation in chloroplast stroma (27). However, purified CpNiFS has only a very low level of Cys desulfurase activity by itself (18). In *in vitro* experiments, the newly identified chloroplast protein CpSufE interacts with CpNiFS and stimulates the Cys desulfurase activity of CpNiFS over 40-fold. Purified CpSufE enhances the Cys desulfurase activity of CpNiFS by affecting both the substrate affinity and Vmax. Indeed the ratio of Vmax/KM was increased 100-fold by CpSufE addition to CpNiFS. Furthermore, the CpNiFS-dependent Fe-S reconstitution activity was found to be enhanced 20-fold by CpSufE. These findings provide at least a partial explanation for the 50 – 80-fold higher reconstitution ability of chloroplast stroma compared to CpNiFS alone (27) and directly connect a SufE-like protein with Fe-S cluster formation. CpSufE and CpNiFS should also be able to interact in *vivo* because both proteins are localized in the stroma and have comparable expression patterns and expression levels. Thus, CpSufE appears to be an important regulator for both Cys desulfurase activity and Fe-S formation in plastids.

The SeCys lyase activity of CpNiFS is not much affected by CpSufE. Therefore plastids have the ability to decompose SeCys and thus prevent the unspecific and toxic incorporation of Se in proteins, regardless of the activity of CpSufE. Indeed, overproduction of CpNiFS (without CpSufE) enhanced Se-tolerance (38).

SufE proteins are evolutionary conserved. Homologues are found in a wide variety of organisms including prokaryotes and eukaryotes. All these proteins have a conserved Cys residue that seems to function in a sulfur transfer pathway, accepting the S atom that is bound as sulfane sulfur to an active site Cys in a type II, SufS-like Cys desulfurase (29, 33). This mechanism seems to be conserved in the chloroplast and the cys residue of CpSufE was also found to be essential for efficient Cys desulfurase activity of CpNiFS. However, compared to bacterial proteins, the plant homologues have some unique properties. The Arabidopsis and rice SufE proteins have an extra C-terminal domain in their mature protein that shows good sequence similarity to *E. coli* BolA. The function of this BolA-like domain is at this point unclear. In *E. coli*, BolA is involved in the regulation of cell division in response to nutrition (34), however the molecular mechanism by which BolA functions is not yet resolved. Recently, a comparison of data from genomic sequences, yeast-two-hybrid experiments, and three-dimensional structures, led to the hypothesis that the BolA protein may be a reductase interacting with a mono-thiol glutaredoxin (35). The bacterial BolA-glutaredoxin pair could be involved in defense against oxidative stress, targets of which are proteins or other organic compounds that are part of membranes or cell walls. The human glutaredoxin Grx2 was found to be a Fe-S protein likely functioning as a redox sensor (39). The 2Fe-2S cluster of holo-Grx2 is degraded under oxidative stress. The resulting apo-Grx2 becomes active, which causes a series of responses including protein de-/glutathionylation, anti-
apoptosis and reduction of low molecular weight disulfides. This leads to the hypothesis that the CpSufE BolA domain may function as a redox sensor via interaction with a glutaredoxin. The Cys desulfurase activity of the CpNifS-CpSufE complex, and even the entire Fe-S biosynthetic machinery may thus be regulated in response to the redox state of the chloroplast stroma. In other studies, a glutaredoxin (Grx5) was reported to be directly required for Fe-S assembly in yeast and animals (40, 41, 42). A defect in Grx5 severely affected Fe-S cluster synthesis, but the wildtype phenotype could be restored by overexpressing other components involved in Fe-S cluster biosynthesis. The function of Grx5 could be to regulate the redox state of important cysteine residues in mitochondrial Nfs1 and/or in the scaffold proteins (41). In analogy, it is therefore also feasible that a glutaredoxin interacting with the BolA domain of CpSufE is directly involved in chloroplastic Fe-S cluster biogenesis, by reducing cysteine residues in the active centers of CpNifS, CpSufE, CpIscA, or Nfu scaffolds. These recent findings raise a great interest to investigate the possible involvement of a glutaredoxin in plastidic Fe-S synthesis. However, the presence of multiple Grx candidates in chloroplasts adds complexity to addressing this question.

We found that CpSufE and CpNifS interact dynamically in vitro, as judged from gelfiltration, native PAGE and affinity chromatography experiments. The two proteins may also interact in vivo in the chloroplast stroma. When stromal proteins were separated by gelfiltration and analyzed by immunoblotting, part of CpSufE (5-10%) eluted at a molecular weight of ~600 kDa, while most of the CpSufE appeared to be in the dimeric form (results not shown). Earlier, CpNifS also was found to elute in part at this molecular weight (27), as did the putative Fe-S scaffold protein CpIscA (22). Thus, all three proteins may be part of the same complex in vivo. The observations that CpSufE is present mostly in dimeric form in the stroma and that maximum stimulation of CpNifS requires a 5-fold excess and not just an equal amount of CpSufE both may be taken to indicate that the interaction is transient in vivo. Remarkably, the mutation of the conserved cysteine to serine in either protein still allows interaction of CpNifS and CpSufE as measured by chromatography coelution, indicating that other amino acids are responsible for the binding. Interestingly, when present in excess, the mutant CpSufEC65S has a dominant negative effect on the activity of CpNifS, competitively inhibiting the activation by WT CpSufE (Fig. 6). This experiment further confirms the concept that the binding and formation of a two-component Cys desulfurase is a prerequisite for CpNifS activation by CpSufE. In contrast to the dominant negative effect of the CpsufE mutant, an excessive amount of mutant CpNifSC388S did not prevent WT CpNifS from being activated by WT CpSufE, possibly because the CpNifSC388S has a lower ability to bind to CpSufE than the WT protein, as suggested by the native PAGE experiments. Together, these observations may be explained by the following proposed reaction mechanism: CpNifS binds the substrate cysteine. CpSufE monomers then bind to dimeric CpNifS and the binding affinity is increased after CpNifS has decomposed the substrate Cys and has a sulfane sulfur bound to its active site - something the CpNifS mutant can not do, and therefore it does not act dominant negative. After transfer of the S to the cysteine of WT CpSufE the protein interaction becomes again reversible for the WT proteins and CpSufE can leave and deliver the S to downstream targets such as scaffolding proteins in vivo. In vitro this acceptor could be the DTT in the reaction mixture. The mutant CpSufE cannot receive the S from CpNifS and therefore is not released as easily, resulting in the observed dominant negative effect.

Purified CpSufE was observed to be a dimer in solution (Fig 4, 7) yet our experiments indicate that CpSufE enters complexes with CpNifS as a monomer. In this context it may be noted that bacterial SufE was observed to be mainly monomeric in solution (43). The proposed changes in affinity and the dynamic behavior of CpSufE during the catalytic cycle imply that conformational changes would occur in CpSufE and possibly CpNifS. The active site cysteine in bacterial SufE is located such that the bound sulfane-sulfur would be shielded from solvent interactions (43). The structure of SufE implies that conformational changes must occur as SufE accepts sulfur from SufS/CpNifS (43). In the homologous plastid protein this arrangement may help protect the sulfur from interaction with reactive oxygen, thus helping make Fe-S formation possible in this oxygenic environment.
In addition to CpSufE, homologues have been found in Arabidopsis for all of the bacterial Suf proteins, and all are predicted to be present in plastids. Therefore, it seems that in the course of evolution plants have retained the entire Suf machinery including SufABCDES for chloroplast Fe-S cluster assembly (for review see: 5). CpNifS is the SufS-like protein, which is a Cys desulfurase supplying sulfur for the Fe-S cluster formation. CpSufE is the SufE-like protein, which is a Cys desulfurase activator as reported in this paper. CplscA is a SufA-like scaffold protein where a transient 2Fe-2S cluster is assembled (22). A similar pattern of gene expression was found for these proteins, supporting the possibility that they may function in the same biochemical process of Fe-S assembly (this work, 22). Chloroplastic SufB, SufC and SufD are homologues of bacterial SufB, SufC and SufD, respectively. They form a complex, which displays an iron-stimulated ATPase activity (23, 24, 25). However, a direct role of CpsufBCD in the Fe-S formation still needs to be demonstrated. The Suf system in bacteria is responsible for Fe-S formation under oxidative stress and iron limitation (12). This may explain why plants contain a Suf-like system for biosynthesis of Fe-S clusters in chloroplasts, as they are oxygen producing compartments. It has been suggested that the S in SufE may be somewhat shielded. Besides Suf-like proteins, chloroplasts also contain Nfu-like scaffolds and the HCF 101 protein for Fe-S cluster biosynthesis (19, 26), suggesting that the Fe-S cluster biogenesis machinery in chloroplasts is more complex than any single operon in bacteria. Because of the barrier formed by the envelope, Fe-S clusters synthesized outside plastids cannot be easily supplied to chloroplasts, which is self-sufficient for the synthesis of its Fe-S clusters. Since plant plastids require a variety of Fe-S cluster types and have at least 20 Fe-S proteins there is perhaps a requirement for multiple-protein components. For instance, Nfu1-3 and CplscA may be alternative scaffolds either for assembling different types of Fe-S clusters or for different physiological conditions. Among these many components of the plastid Fe-S cluster biosynthesis machinery, CpNifS and CpSufE hold a central and apparently essential position. Analysis of T-DNA insertion knockout lines for CpSufE indicate that knockout of CpSufE is seed-lethal. Offspring from a heterozygous CpSufE knockout gave rise to seeds that showed 100% germination, but a 2:1 heterozygous : wildtype ratio, with complete absence of homozygous knockouts (Ye, Abdel-Ghany, Anderson, Pilon-Smits and Pilon, unpublished results).

In conclusion, the new results presented here reveal a new key component of the iron-sulfur biogenesis machinery in plant chloroplasts. These results are of significance since still very little is known about how chloroplast iron-sulfur clusters are made, despite their vital importance for photosynthesis and assimilatory nitrogen and sulfur assimilation. Photosynthesis is the process that determines plant productivity and ultimately drives life on earth. Its importance for biology cannot be overstated. Also, plant iron status is of importance for human health, since billions of people worldwide are suffering from iron deficiency and most of the dietary iron in developing countries is derived from vegetarian food sources.

REFERENCES


**FOOTNOTES**

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The abbreviations used are: At, *Arabidopsis thaliana*; Cp, chloroplast; Fd, ferredoxin; Cys, cysteine; SeCys, selenocysteine; CBB, Coomassie Brilliant Blue; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WT, wild type; PLP, pyridoxal 5' phosphate; DTT, dithiothreitol.

**FIGURE LEGENDS**

Fig. 1. A, Sequence alignment of SufE-like proteins from various organisms. The predicted transit sequence of Arabidopsis CpSufE is underlined. Identical residues are shaded in gray. The conserved cysteine at position 131 in Arabidopsis precursor CpSufE (or position 65 in mature CpSufE) is boxed. Species used are as follows. At: *Arabidopsis thaliana*, Os: *Oryza sativa*, Sy: *Synchocystis* sp. PCC 6803, Ec: *Escherichia coli*, Er: *Erwinia chrysanthemi*, Ag: *Agrobacterium tumefaciens*. B, Schematic domain structure of CpSufE. TP: targeting peptide.

Fig. 2. Localization of CpSufE in chloroplasts. A, Subcellular localization of GFP and CpSufE-GFP fusion proteins. CpSufE-GFP fusion proteins were expressed in Arabidopsis protoplasts, and observed using a confocal laser-scanning microscope. Green fluorescence, red chlorophyll fluorescence and overlays of green and red signals are shown. B, Immunoblotting analysis of total leaf homogenate (TH) and chloroplast stroma (St). Homogenate and stroma, containing 2.5 µg chlorophyll for each, were separated by 12.5% SDS-PAGE, blotted to nitrocellulose after which CpSufE was detected with a specific antibody. Pure CpSufE protein served as a positive control. The large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) was detected with corresponding antibody to evaluate equal loading.

Fig. 3. Expression analysis of CpSufE. A, Immunoblot analysis of CpsufE and CpNifS expression in different tissues. Each fraction represents 20 µg of proteins. B, RNA blot of CpSufE in different tissues. Ten micrograms of total RNA from different tissues was separated by electrophoresis, transferred to Hybond N+ membrane, and probed with a 32P-labeled 900 bp CpSufE cDNA (top). An ethidium bromide-stained agarose gel was used to verify equal loading (bottom). C, Immunoblot of CpSufE in homogenate from light/dark-grown Arabidopsis. Twenty micrograms of leaf homogenate proteins from light- or dark-grown Arabidopsis were separated by electrophoresis, transferred to nitrocellulose membrane, and probed with CpSufE antibody. Arabidopsis cytosolic Hsp70 was used to evaluate equal loading. The large subunit of RuBisCo was used as a control of light-regulation. D, RNA blot of CpSufE in homogenate from light/dark-grown Arabidopsis. Ten micrograms of total leaf homogenate RNA from light or dark-grown Arabidopsis was separated by electrophoresis, transferred to Hybond N+ membrane, and probed with a 32P-labeled 900 bp CpSufE cDNA (top). An ethidium bromide-stained agarose gel was used to show loading (bottom).
Fig. 4. Purification of CpSufE. A, Expressed region of CpSufE in the pET28a vector. Both nucleotide and amino acid sequences are used to show the expressed region of CpSufE in pET28a. The His tag, thrombin cleavage site, and mature CpSufE in the expressed protein are labeled. The restriction sites of NdeI and BamHI for subcloning of CpSufE are labeled in italics. B, SDS-PAGE analysis of CpSufE at different purification stages. The gel was stained with Coomassie Brilliant Blue. lane 1, total cell lysate of E. coli BL21 (DE3) / codon+ expressing His-tagged CpSufE; lane 2, eluate of 1 M imidazole from nickel column containing His-tagged CpSufE; lane 3, eluate of ~ 0.3 M NaCl in linear salt gradient from Resource-Q column after thrombin treatment. C, Elution profile (A280) of purified CpSufE from a Superdex 200 gelfiltration column.

Fig. 5. CpSufE stimulates Cys desulfurase activity 40-fold but reduces SeCys lyase activity of CpNifS. A, Cys desulfurase activity of CpNifS (2.5 μM) mixed with CpSufE in molar ratios ranging from 0 to 20 (CpSufE : CpNifS). B, Selenocysteine lyase activities of proteins and protein combinations present at 2.5 μM. C, Cysteine desulfurase activities of proteins and protein combinations present at 2.5 μM. Data are the means ± SE of 3 experiments.

Fig. 6. Dominant negative effect of CpSufEC65S. Cysteine desulfurase activity was assayed for the protein mixtures at the indicated protein concentrations. Data are the means ± SE of 3 experiments.

Fig. 7. CpSufE forms complexes with CpNifS. A, Superdex-200 gelfiltration elution profiles (A280). trace 1, 15 μg CpSufE; trace 2, 17 μg CpNifS; trace 3, mixture of 15 μg CpSufE and 17 μg CpNifS; trace 4, mixture of 15 μg CpSufEC65S and 17 μg CpNifS. B, SDS-PAGE analysis of gelfiltration fractions described in panel A. Aliquots of the indicated fractions were collected and separated on SDS-PAGE gels followed by staining with Coomassie Brilliant Blue. The peak fraction in each gelfiltration run is marked by a asterisk. C, Superdex-200 gelfiltration elution profile of a mixture of 200 μg CpSufE and 200 μg CpNifS. D, Separation of CpSufE and CpNifS proteins and mixtures of CpSufE-CpNifS complexes on a 10% native PAGE gel. Proteins were visualized by staining with Coomassie Brilliant Blue.

Fig. 8. Separation of CpSufE-CpNifS complexes on a Ni-NTA-column. A, Ni-NTA-column coelution of His-tagged CpSufE and CpNifS. 100 μg His-tagged CpSufE (WT or mutant) and 100 μg CpNifS (WT or mutant) were mixed and loaded on a 0.5 ml Ni-IDA agarose column, followed by a series of washes as described in Materials and Methods. The 1 M imidazole eluates were collected and separated on a 12.5% SDS-PAGE gel followed by staining with Coomassie Brilliant Blue. Purified His-tagged CpSufE, CpNifS and mutant proteins were run on a same gel as controls. B, Ni-NTA-column coelution of His-tagged CpSufE, cleaved CpSufE (or CpSufEC65S) and CpNifS. The mixture of the three proteins was loaded and eluted from a Ni-NTA-column as described in Materials and Methods. The 1M imidazole eluates were separated on a 15% SDS-PAGE gel. Group I: His-tagged CpSufE, cleaved WT CpSufE, and CpNifS. Group II: His-tagged CpSufE, cleaved CpSufEC65S mutant, and CpNifS. C, a model for explaining the presence of cleaved CpSufE in the 1M imidazole eluate as shown in panel B. E: CpSufE; S: CpNifS; His: 6-Histidine-tag. The His-tagged CpSufE dimer and the cleaved CpSufE dimer split into monomers. One His-tagged CpSufE monomer and one cleaved CpSufE monomer both bind to a CpNifS dimer, forming a heterotetrameric complex that binds to the Ni-column. D, a model for the formation of CpSufE-CpNifS complexes. A CpSufE dimer splits into two monomers. One of the CpSufE monomers binds to a dimeric CpNifS, leading to a trimeric NifS2-SufE complex, which can acquire another CpSufE monomer, resulting in a tetrameric complex.

Fig. 9. CpSufE enhances the Fe-S reconstitution in ferredoxin 20-fold. Protein(s) were incubated with 50 mM Tricine-NaOH, pH 7.5, 5 mM DTT, 1 mM L-Cys, 1 mM ferrous ammonium sulfate, 20 μM PLP for
30 min, followed by addition of 30 μg of apo-Fd. After 20 min, the reconstituted holo-Fd was measured by HPLC. Data are the means ± SE of 3 experiments.
### Table 1. Sequence of oligonucleotides used for cloning and plasmid constructions

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<td>Sal I</td>
</tr>
<tr>
<td>SufE-GFP-R</td>
<td>CATGCCATGGCCTACGAGGAGTCTTTGC</td>
<td>Nco I</td>
</tr>
<tr>
<td>SufE-GFP-RT</td>
<td>CATGCCATGGGCTGTCGTTAGGTGAAGCTCT</td>
<td>Nco I</td>
</tr>
<tr>
<td>NifSC388-F</td>
<td>AAGGCAGACCAAACCTCCGCACGAGCCACTCCA</td>
<td>_</td>
</tr>
<tr>
<td>NifSC388S-R</td>
<td>TGGAGTCGTGTGCGGAGGTGCTCAGTGGAACTCCT</td>
<td>_</td>
</tr>
<tr>
<td>NFS-mat</td>
<td>See (18)</td>
<td></td>
</tr>
<tr>
<td>NFS-B2</td>
<td>See (18)</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. CpSufE changes kinetic properties of CpNifS. K_M values toward cysteine and selenocysteine, and reaction velocities (V_{max}) were determined over the range of 0.01 to 20 mM substrate, as described in the Materials and Methods. The program Enzfitter (Biosoft, Cambridge, UK) was used to calculate K_M and V_{max} values. Data are the means and standard error of 3 experiments.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>CpNifS</th>
<th>CpNifS + CpSufE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_M (mM)</td>
<td>V_{max} (units/mg)</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.10±0.02</td>
<td>0.0013±0.0002</td>
</tr>
<tr>
<td>Selenocysteine</td>
<td>2.90±0.36</td>
<td>2.44 ± 0.07</td>
</tr>
</tbody>
</table>
Figure 2

A. 

<table>
<thead>
<tr>
<th>GFP</th>
<th>green fluorescence</th>
<th>red fluorescence</th>
<th>red and green fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AtCpSufE-TP-GFP (1-66)</th>
<th>green fluorescence</th>
<th>red fluorescence</th>
<th>red and green fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AtCpSufE-FL-GFP</th>
<th>green fluorescence</th>
<th>red fluorescence</th>
<th>red and green fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. 

<table>
<thead>
<tr>
<th>TH St 1 5 25 50 ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpSufE</td>
</tr>
<tr>
<td>RuBisCo (large subunit)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pure CpSufE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH St 1 5 25 50 ng</td>
</tr>
<tr>
<td>CpSufE</td>
</tr>
<tr>
<td>RuBisCo (large subunit)</td>
</tr>
</tbody>
</table>
Figure 3

A. 

<table>
<thead>
<tr>
<th>kDa</th>
<th>leaf</th>
<th>stem</th>
<th>flower</th>
<th>root</th>
</tr>
</thead>
<tbody>
<tr>
<td>48.8</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>37.1</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>

CpSufE
CpNifS

B. 

<table>
<thead>
<tr>
<th>leaf</th>
<th>stem</th>
<th>flower</th>
<th>root</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>

CpSufE

28S
18S

C. 

<table>
<thead>
<tr>
<th>kDa</th>
<th>Dark</th>
<th>Light</th>
</tr>
</thead>
<tbody>
<tr>
<td>48.8</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>37.1</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>48.8</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>37.1</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>

CpSufE
Hsp70
RuBisCo

D. 

<table>
<thead>
<tr>
<th>Dark</th>
<th>Light</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>

CpSufE

28S
18S
Figure 4

A. ATGGGCAAGCCATCATCATCATCATCATCAGCGGGCTGCCGCCGGCGCGCGCGCGCCATATGGCTTCATCA------GAGGTGTGAGGATCCGAATTC

MetGlySerSerHisHisHisHisHisHisSerSerGlyLeuValProArgGlySerHisMetAlaSerSer-----GluValstop

His6 tag

B. B. kDa

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>64.2</td>
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<td>48.8</td>
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<td>37.1</td>
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</tr>
<tr>
<td>25.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C. Absorption 280 nm (mAU)

retention time (min)

13.0 14.0 15.0 16.0 17.0 18.0 19.0 20.0 21.0

dimeric CpSufE

Nde I BamHI

thrombin↑ CpSufE
Figure 5

A. Cys desulfurase activity (µmol sulfide/min/mg) vs. molar ratio (CpSuF/E/CpNiF)

B. Secys lyase activity (µmol selenide/min/mg) for different combinations of proteins:
   - CpNiF
   - CpNiF+CpSuF
   - CpNiF+CpSuF+Ec65s
   - CpNiF+CpSuF+CpSuF+Ec65s

C. Cys desulfurase activity (µmol sulfide/min/mg) for different combinations of proteins:
   - CpNiF
   - CpNiF+CpSuF
   - CpNiF+CpSuF+Ec65s
   - CpNiF+CpSuF+CpSuF+Ec65s
Figure 6

CysD activity (µmol sulfide/min/mg)

<table>
<thead>
<tr>
<th></th>
<th>WT CpNifS</th>
<th>Mut CpNifS</th>
<th>WT CpSufE</th>
<th>Mut CpSufE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concen</td>
<td>1.5 µM</td>
<td>7.5 µM</td>
<td>1.5 µM</td>
<td>7.5 µM</td>
</tr>
<tr>
<td>Activity</td>
<td>0.05</td>
<td>0.01</td>
<td>0.06</td>
<td>0.02</td>
</tr>
</tbody>
</table>
Figure 8

A. 

<table>
<thead>
<tr>
<th></th>
<th>loading controls</th>
<th>imidazole eluates</th>
</tr>
</thead>
<tbody>
<tr>
<td>His-CpSufE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>His-CpSufEc65s</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CpNifS</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CpNifSc388s</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

B. 

<table>
<thead>
<tr>
<th></th>
<th>NiIFS tagged SuF</th>
<th>1 M imidazole elutes I</th>
<th>1 M imidazole eluates II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cleaved SuF</td>
<td>+cys -cys</td>
<td>+cys -cys</td>
</tr>
</tbody>
</table>

C. 

D. 

[Diagram showing molecular interactions with labels: His, His-IDA resin, etc.]
Figure 9

Holo-fd (µg)

Cp CafE (4µg)
Cp NifS (5µg)
Cp CafE (4µg) + Cp NifS (5µg)
Cp NifS (100µg)