Characterization of Arabidopsis thaliana SufE2 and SufE3
FUNCTIONS IN CHLOROPLAST IRON-SULFUR CLUSTER ASSEMBLY AND NAD SYNTHESIS

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In this study we characterize two novel chloroplast SufE-like proteins from Arabidopsis thaliana. Other SufE-like proteins, including the previously described A. thaliana CpSufE, participate in sulfur mobilization for Fe-S biosynthesis through activation of cysteine desulfurization by NiFS-like proteins. In addition to CpSufE, the Arabidopsis genome encodes two other proteins with SufE domains, SufE2 and SufE3. SufE2 has plastid targeting information. Purified recombinant SufE2 could activate the cysteine desulfurase activity of CpNiFS 40-fold. SufE2 expression was flower-specific and high in pollen; we therefore hypothesize that SufE2 has a specific function in pollen Fe-S cluster biosynthesis. SufE3, also a plastid targeted protein, was expressed at low levels in all major plant organs. The mature SufE3 contains two domains, one SufE-like and one with similarity to the bacterial quinolinic acid synthase, NadA. Indeed SufE3 displayed both SufE activity (stimulating CpNiFS cysteine desulfurase activity 70-fold) and quinoline synthase activity. The full-length protein was shown to carry a highly oxygen-sensitive (4Fe-4S) cluster at its NadA domain, which could be reconstituted by its own SufE domain in the presence of CpNiFS, cysteine and ferrous iron. Knock-out of SufE3 in Arabidopsis is embryotoxic. We conclude that SufE3 is the NadA enzyme of A. thaliana, involved in a critical step during NAD biosynthesis.

In plants, Fe-S proteins are particularly important for photosynthesis. In the chloroplast thylakoid membrane, Fe-S cluster proteins function in photosynthetic electron transport leading to the production of ATP and NADPH (7). These molecules provide the energy and reducing power for photosynthetic carbon reduction and assimilation, as well as for sulfur and nitrogen reduction and assimilation, which require Fe-S prosthetic groups as cofactors for sulfite reductase and nitrite reductase (for a review, see Ref. 8).

Fe-S cluster biogenesis can be divided into elemental sulfur formation, iron acquisition, assembly of iron and sulfur into a cluster, and cluster insertion into apoproteins (2, 3). Briefly, sulfur is mobilized from cysteine by the action of a pyridoxal phosphate-dependent cysteine desulfurase enzyme (9, 10). The sulfur is combined with iron atoms, of which the source is still unknown, on a scaffold protein. There, a transient Fe-S cluster is assembled before insertion into apoproteins to form functional Fe-S proteins (11–15).

In bacteria three main Fe-S assembly systems have been described which are termed NIF (nitrogen fixation), ISC2 (Fe-S cluster) and SUF (mobilization of sulfur) (9, 16–18); each system contains a cysteine desulfurase. While the NIF operon has a specialized function in nitrogen fixation in certain bacteria, the ISC operon is thought to be involved in the general Fe-S cluster biosynthesis pathway (9). The SUF system appears to represent an alternative pathway for the assembly of Fe-S clusters under oxidative stress and iron limitation (16, 19).

In plants, chloroplasts have their own Fe-S cluster biogenesis machinery (20, 21). It is likely that the redox conditions in the chloroplast are particularly challenging for the synthesis and maintenance of Fe-S cluster proteins, because Fe-S clusters are very sensitive to oxygen and photosynthesis produces oxygen (1). Therefore, chloroplasts must have unique mechanisms better suited for Fe-S cluster formation under oxidizing conditions and/or mechanisms to replace or repair oxidatively damaged clusters. It is therefore not surprising that, based on sequence similarities, the plastid Fe-S biosynthesis components identified to date are most related to the bacterial Suf cluster genes, known to function under oxidative stress (4).

Fe-S clusters are one of the most common and functionally diverse types of prosthetic groups that can be found in all living organisms (1). Fe-S proteins are involved in processes such as electron transport, redox and non-redox catalysis, sensing of environmental stimuli, DNA repair, and regulation of gene expression (2–4). Although Fe-S clusters are derived from two elements on earth, they are not formed spontaneously in cells but arise by controlled biosynthesis of the most abundant elements on earth (5, 6).

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2 The abbreviations used are: ISC, Fe-S cluster; QA, quinolinic acid; QS, quino-linate synthase; DHAP, dihydroxy acetone phosphate; GFP, green fluorescent protein; HPLC, high pressure liquid chromatography; RT, reverse transcription.
Characterization of the chloroplastic Fe-S machinery started with the discovery and characterization of the cysteine desulfurase CpNifS, also known as NFS2 (22, 23). The cysteine desulfurase activity of CpNifS is required for Fe-S cluster formation in the chloroplast protein ferredoxin in vitro (24). Other components of the plastidic Fe-S cluster biosynthetic machinery are the putative scaffold proteins CpNfu1–3 (25–27) and CplscA (28, 29). In addition, several other Suf-type components may assist in Fe-S formation in plastids (30–32). The CpSufBCD complex is an ATCase that may be involved in providing iron or transferring the Fe-S cluster from the scaffold protein to the target protein (31, 32).

Arabidopsis CpNifS is a class II cysteine desulfurase and has only a low level of Cys desulfurase activity in vitro (23), similar to its bacterial homologue SufS. The cysteine desulfurase activity of Escherichia coli SufS was enhanced severalfold in the presence of bacterial SufE; SufE alone had no cysteine desulfurase activity (33, 34). The conserved cysteine of bacterial SufE is critical for the stimulation of cysteine desulfurase activity of SufS (33, 34). In addition, SufE, a homodimeric protein without cofactor, was shown to tightly bind to SufS to form a heterotetrameric complex (33). Homologues of SufE are found in a wide variety of organisms, including prokaryotes and eukaryotes. All of these proteins have a conserved cysteine residue that is proposed to function as a mediator during sulfur transfer. After cysteine desulfurization by NifS/SufS-like proteins, the conserved cysteine of SufE abstracts the sulfur atom from the SufS persulfide and thus becomes an intermediate persulfide (33–35). Then, from the latter, sulfur is transferred to an acceptor protein where it serves for synthesis of a sulfur-containing compound such as a Fe-S cluster.

Recently a SufE-like protein (At4g26500) was discovered in Arabidopsis chloroplasts that can stimulate the cysteine desulfurase activity of CpNifS (36, 37). This protein, which was originally called SufE/CpSufE (36, 37), will be referred to as SufE1 from hereon. Arabidopsis SufE1 represents an evolutionary conserved SufE protein that can complement the growth defects in SufE-deficient E. coli (36). Interestingly, SufE1 was localized to both chloroplasts (36, 37) and mitochondria (36). It binds to and forms a heterotetrameric complex with CpNifS, stimulating its cysteine desulfurase activity 40-fold and increasing the substrate affinity of CpNifS toward cysteine (37). In vitro reconstitution assays showed that the capacity of CpNifS to assemble 2Fe-2S clusters in ferredoxin was improved 20-fold in the presence of SufE1 (37). The conserved cysteine residue (Cys<sup>50</sup>) of Arabidopsis SufE1 is essential for this stimulation and is probably the acceptor site for sulfur, in analogy with E. coli SufE (37). The embryo-lethal phenotype of T-DNA disruption lines of Arabidopsis SufE1 demonstrated that it is an essential protein (36, 37).

Genomic database searches revealed that next to SufE1, Arabidopsis contains two more genes that encode proteins with SufE-like domains. In this study, we characterize these two proteins, which we named SufE2 and SufE3. SufE2 consists of only a SufE-domain and seems to function in pollen. SufE3 is a two-domain protein consisting of an N-terminal SufE domain linked to a C-terminal domain with homology to bacterial NadA. E. coli NadA is involved in the biosynthesis of NAD, a cofactor in numerous essential redox biological reactions. In all living organisms, NAD derives from quinolinate acid (QA) (38, 39). In most eukaryotes studied to date, QA is produced via the degradation of tryptophan whereas in E. coli it is synthesized from L-aspartate and dihydroxyacetone phosphate (DHAP) as the result of the concerted action of two enzymes, L-aspartate oxidase encoded by the nadB gene, and quinolinate synthase (QS), encoded by the NadA gene (40, 41). Recent studies showed that E. coli NadA is an Fe-S enzyme with a 4Fe-4S cluster absolutely required for activity (42, 43). As in E. coli, in Arabidopsis quinolinate acid is synthesized in the L-aspartate pathway (44). During the course of this work, the Arabidopsis quinolinate synthase was shown to be localized to chloroplasts and to complement an E. coli mutant defective in the corresponding NadA gene (44), but the activity was not demonstrated in vitro. Here, we show that this protein, which we call SufE3, has both SufE and QS activity, the latter of which depends on a highly oxygen-sensitive 4Fe-4S cluster in the NadA domain. The SufE domain on the same protein can reconstitute this cluster through interaction with CpNifS.

**EXPERIMENTAL PROCEDURES**

**Plant Material—** Arabidopsis (Arabidopsis thaliana (L.) Heynh.) ecotype Colombia was used for all studies. Plants were grown in soil at 22 °C with a 16 h/8 h light-dark cycle.

**Plasmid Construction and Expression Analysis—** Primer sequences are listed in Table 1. cDNA clones for SufE2 and SufE3 were obtained from the Arabidopsis Biological Resource Center, Ohio State University (clone numbers 33B2 and U09473, respectively). The A. thaliana SufE2 (At1g67810) and SufE3 (At5g50210) coding sequences were amplified by PCR using cDNA clones as a template. For SufE2 expression in E. coli, the mature sequence of SufE2 was amplified using primers SufE2-Bam and SufE2-Nde. The PCR product was digested with BamHI and NdeI and then ligated into pET28a (Novagen, Madison, WI), for expression as His<sub>6</sub>-tagged protein. The mature sequence of SufE3 was amplified using primers SufE3-mature-Nco and SufE3-mature-Xba. The PCR product was digested with Ncol and XbaI and then ligated into pBAD/Myc-HisB (Invitrogen) to produce pSufE3 for expression as Myc-His<sub>6</sub>-tagged protein. The same construct was used for complementation analysis of the E. coli ΔnadA deletion mutant. To change the single cysteine to serine in the SufE domain of SufE3, recombinant PCR was performed. In the first round, two fragments were amplified with primer set SufE3-R-C132S and T7 and primer set SufE3-F-C132S and T7 terminator, respectively (Table 1). pSufE3 was used as a template. Two products from the first round of PCR were fused together and amplified with primers SufE3-mature-Nco and SufE3-mature-Xba (Table 1). The resulting PCR product was digested with Ncol and XbaI and then subcloned into pBAD/Myc-HisB expression vector to produce plasmid pSufE3<sub>C132S</sub>, also used for complementation analysis of the E. coli ΔnadA deletion mutant. For the expression in E. coli, the SufE domain of SufE3 was amplified using the primers SufE-dom-Bam and SufE-dom-Nde. The PCR product was digested with BamHI and NdeI and then ligated into expression vector pET28a. For green fluorescent protein (GFP) localization studies, the full-length protein
sequence of SufE2 was amplified with flanking primers SufE2-GFP-Sal and SufE2-GFP-Nco, and SufE3 was amplified with the primers SufE3-GFP-Sal and SufE3-GFP-Nco. The PCR products of SufE2 and SufE3 were digested with SalI and NcoI and then inserted into the GFP reporter plasmid 35S-GFP (S65T) (45).

For Northern blot analysis, total RNA was isolated from different tissues (roots, leaves, stems, and flowers) by the TRIzol reagent method (Invitrogen). Ten microgram of total RNA was separated on a 1% (w/v) agarose gel containing 4% (w/v) formaldehyde, transferred to a nylon membrane, and probed with 32P-labeled 18S rRNA fragment as a loading control.

For RT-PCR, one µg of DNase-treated total RNA from different tissues (roots, leaves, stems, and flowers) was used to synthesize first-strand cDNA with an oligo(dT) primer and avian myeloblastoma virus reverse transcriptase in a 20-µl volume. One µl of this template was used for PCR amplification as follows: sense, SufE2RT, and antisense, SufE2RT-rev, primers were used to amplify a 575-bp sequence from different cDNAs. Actin2-F and Actin2-R primers were used to amplify a 477-bp sequence from Arabidopsis cDNA as described (23). The PCR reaction mixtures were resolved in a 1% agarose gel.

Sequence Analysis and Alignments—Sequence analysis was performed using expert sequence analysis software (DNASTAR, Madison, WI). Searches for sequence similarity were performed using the BLAST network service provided by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Sequence alignment was performed using the MegAlign tool of DNASTAR.

Subcellular Localization of GFP Fusion Proteins—For transient expression, the precursor sequences of SufE2 and SufE3 were cloned into the 35S-GFP (S65T) vector (45) under the control of the CaMV 35S promoter to generate fusions with a C-terminal GFP. These constructs were expressed in Arabidopsis protoplasts as described (47). Expressed proteins were observed under a confocal laser scanning fluorescence microscope. GFP was excited at 460–500 nm, whereas chlorophyll fluorescence was excited at 510–560 nm.

Complementation of E. coli Mutants—Plasmids that contained the mature regions of SufE2 or SufE3-C132S in pBAD/Myc-HisB were introduced into E. coli ΔNadA which has a disruption in the NadA gene encoding quinolinate synthase (48). Plasmid containing strains were maintained on LB medium with kanamycin. Colonies were then grown on M9 minimal medium supplemented with 0.4% glycerol, 0.02% arabinose, with or without 12.5 µg/ml nicotinic acid. c-Myc monoclonal antibody was purchased from Clontech (Mountain View, CA). Immunoblotting was performed using a standard protocol as described by (23).

Characterization of T-DNA Knock-out Lines—T-DNA insertion line (SALK_075260) for SufE3 was provided by the SALK institute (49) and obtained from the Arabidopsis Biological Resource Center (ABRC), Ohio State University. To determine the position of the T-DNA insert, a DNA fragment was amplified by PCR using primers specific for the T-DNA left border and for the flanking SufE3 sequence; the nucleotide sequence of the resulting product was determined. To determine the genotype of plants with respect to the T-DNA insertion (homozygous or heterozygous) SufE3-specific primers that flanked the insertion point were used to identify by PCR the presence of a wild type allele, whereas a SufE3-primer in combination with a T-DNA left border primer was used to identify the mutant allele. Using PCR analysis, only wild type individuals or plants heterozygous for T-DNA insertions in SufE3 were observed in a 1:2 ratio in the offspring of heterozygous plants. Growth analysis on kanamycin plates indicated that kanamycin resistance also segregated in a 1:2 ratio. Furthermore, PCR analysis showed that all kanamycin resistant plants had a T-DNA insertion in SufE3. These data indicate the presence of a single T-DNA insertion in this knock-out line.
Characterization of Arabidopsis SufE2 and SufE3

Protein Purification—For purification of the SufE domains of SufE2 and SufE3, respectively, plasmid pET28a containing either the sequence for the SufE domain of SufE2 or of SufE3 fused to an N-terminal His-tag were transformed into E. coli BL21 (DE3) codon” (Stratagene, La Jolla, CA). Two liters 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 A₆₀₀ of 0.5 and expression was induced by addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside followed by incubation for 3 h at 37 °C. The SufE2 and SufE3 domains were purified by the procedure developed by Ye et al. (37) to purify SufE1. Following this procedure, SufE2 needed further purification by cation exchange chromatography using a 10 × 1 cm SP Sepharose column (Amersham Biosciences) equilibrated with 25 mM sodium phosphate buffer. The SufE2 protein, obtained after HPLC, was mixed with an equal volume of 25 mM sodium phosphate buffer before application to the column. The column was washed with 4 column volumes of 25 mM sodium phosphate buffer, pH 7.0, and then the SufE2 protein was eluted with 500 mM NaCl in sodium phosphate buffer, pH 7.0. The purified proteins were dialyzed against 25 mM Tris-HCl, pH 7.0, and then the SufE2 protein was eluted with 500 mM NaCl in sodium phosphate buffer, pH 7.0. The purified proteins were dialyzed against 25 mM Tris-HCl, pH 7.0, and stored at −80 °C.

E. coli NadA protein was obtained as described (42). Wild type CpnNifs and the mutant form CpnNifsC₃₈₈₈₈ were purified as described (23, 37). Purification of SufE3 mature protein for quinolinate synthase activity studies was done as follows. For the anaerobic purification of full-length SufE3, a culture of S. enterica SufE3 was grown aerobically in minimal M₉ Fe-enriched medium (35) to purify SufE1. Following this procedure, SufE2 needed further purification by cation exchange chromatography using a 10 × 1 cm SP Sepharose column (Amersham Biosciences) equilibrated with 25 mM sodium phosphate buffer. The SufE2 protein, obtained after HPLC, was mixed with an equal volume of 25 mM sodium phosphate buffer before application to the column. The column was washed with 4 column volumes of 25 mM sodium phosphate buffer, pH 7.0, and then the SufE2 protein was eluted with 500 mM NaCl in sodium phosphate buffer, pH 7.0. The purified proteins were dialyzed against 25 mM Tris-HCl, pH 7.0, and stored at −80 °C.

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Enzyme Assays—Cysteine desulfurase activity was measured as described by Ye et al. (37). NadA enzymatic activity was assayed under anaerobic conditions, inside a glove box at 37 °C, and the time-dependent formation of QA was measured directly by HPLC. The assay contained a final volume of 1 ml, buffer D (50 mM Na-HEPES, 100 mM KCl, pH 7.5), 1 mM DHAP, 33 mM L-aspartate, 25 mM fumarate, 15 μM of either apo-SufE3 or Fe-S-containing SufE3 protein (as-isolated or chemically reconstituted). After a 5-min preincubation of the assay mixture at 37 °C, the reaction was initiated by the addition of 1.5 μM FAD-containing NadB. For the quantification of quinolinic acid, aliquots (100 μl) were removed at different times and added to 5 μl of 2 N H₂SO₄ to quench the reaction. Precipitated proteins were removed by centrifugation at 15,000 rpm for 15 min. 25 μl of the supernatant was injected directly onto the HPLC. The QA produced was analyzed and quantified by a HP-1100 HPLC system after injection onto a Tosoh TSK Gel ODS-120T (4.6 mm × 15 cm) column. The column was eluted with buffer E (0.03% trifluoroacetic acid, pH 2.4) at a flow rate of 0.5 ml/min. QA was detected by its absorbance at 260 nm and was eluted with a retention time of 9.1 min. After 20 min of elution the column was regenerated by applying a linear gradient from 0 to 100% acetonitrile in 0.03% trifluoroacetic acid at 0.5 ml/min over 10 min and re-equilibrated before injection of the next sample. Authentic quinolinic acid (Sigma) was used to generate a standard curve from 0–40 nmol.

Analysis of Total Protein, Protein-bound Iron, and Labile Sulfide—Protein concentration was determined by the method of Bradford (50). Protein-bound iron was determined under reducing conditions with bathophenanthroline disulfonate after acid denaturation of the protein (51) and labile sulfide by the method of Beinert (52).

Mössbauer Spectroscopy—E. coli NadA mutant strain MG1655 (ΔNadA) was transformed with a pBAD vector containing the A. thaliana SufE3 gene. Cells were grown aerobically in minimal M₉ Fe-enriched medium (35) supplemented with 2 mM MgSO₄, 0.4% glucose, 2 μg/ml thiamine, 1 mM CaCl₂, and protein expression was induced by addition of 0.2% arabinose. Anaerobic purification of SufE3 was performed as described above. 10 mg of pure protein were obtained from 10 liter of culture. ⁵⁷Fe-Mössbauer spectra were recorded in 400 μl cuvettes containing 250 μl protein. Spectra were recorded on a spectrometer operating in constant acceleration mode using an Oxford cryostat that allowed temperatures from 4.2 to 300 K and a ⁵⁷Co source in rhodium. Isomer shifts are reported relative to metallic iron at room temperature.
Characterization of Arabidopsis SufE2 and SufE3

**EPR Spectroscopy**—Spectra were recorded on a Bruker EMX (9.5 GHz) or ER200D EPR spectrometer equipped with an ESR 900 helium flow cryostat (Oxford Instruments). Double integrals of the EPR signals and spin concentration were obtained with the Win-EPR software using the spectrum of a 1 mM Cu(EDTA) standard recorded under non-saturating conditions.

**RESULTS**

**SufE Genes and Their Sequence Characteristics**—Earlier, a SufE-like sequence (At4g26500) was identified by a BLAST search within the Arabidopsis genome data base, and called CpSufE/SufE (36, 37). This protein was shown to be targeted to the chloroplast stroma and to increase CpNiFS desulfurase activity 40-fold (37). In the current study, we refer to At4g26500 as SufE1 for two reasons. One, Xu and Möller (36) found that it also localizes to mitochondria and, second, in this study we characterize two other SufE-like proteins in Arabidopsis. Using *E. coli* SufE as a query sequence, we found two more SufE-like proteins encoded in the Arabidopsis genome, At1g67810 (named SufE2) and At5g50210 (named SufE3) (Fig. 1 and supplemental Fig. 1A).

The genomic sequence of Arabidopsis SufE2 contains a single predicted exon. A cDNA containing the full coding sequence was obtained from The Arabidopsis Information Resource (TAIR) data base. Sequence analysis of the cDNA confirmed the presence of a single uninterrupted coding sequence in the genome. The open reading frame is predicted to encode a full-length precursor protein of 259 amino acids, including a putative chloroplast targeting peptide of 41 amino acids. The predicted mature polypeptide is 215 amino acids long with a molecular mass of 25 kDa and an isoelectric point of 6.9. SufE2 has sequence similarity to previously characterized SufE proteins (Fig. 1 and supplemental Fig. 1A).

The open reading frame of SufE3 is predicted to encode a full-length precursor protein of 717 amino acids including a 69 amino acid putative chloroplast targeting peptide. The predicted mature polypeptide is 646 amino acids long with a molecular mass of 69 kDa and an isoelectric point of 6.2. In SufE3, the N-terminal half of the predicted mature protein resembles a SufE-like region (Fig. 1 and supplemental Fig. 1A). This predicted SufE domain is 150 amino acids in length with a molecular mass of 17.9 kDa and an isoelectric point of 5.1. The C-terminal 484 amino acids of SufE3 have good sequence similarity to the SufE domain of these Arabidopsis SufE1–3 proteins (37). A sequence alignment was performed to determine the similarity of the SufE domain of these Arabidopsis SufE1–3 proteins and *E. coli* SufE (supplemental Fig. 1B). All SufE homologues show conservation in the mature protein region including a highly conserved single cysteine which was shown to be critical for function in bacterial SufE and also in Arabidopsis SufE1 (37). In SufE1, the SufE domain corresponds to the N-terminal half of the predicted mature protein (Fig. 1). The C-terminal domain of SufE1 shows good sequence similarity to bacterial BolA (for sequence similarity, see Ref. 37).

**Subcellular Localization and Expression of SufE2 and SufE3**—The prediction program TargetP predicts that SufE2 and SufE3 are targeted to the plastids (53). To determine the subcellular distribution of these Arabidopsis proteins experimentally, we constructed fusions between the precursor sequences and GFP. GFP expressed by itself was used as a control. Constructs were separately introduced into Arabidopsis protoplasts and the localization in cells was studied using confocal laser-scanning microscopy. Examination of non-fused GFP showed localization to the cytoplasm as expected (Fig. 2, top panel). The fusions for both SufE2 and SufE3 showed localization to the chloroplasts, as indicated by the overlay of green fluorescence and red autofluorescence from chlorophyll (Fig. 2, middle and bottom panel, respectively).

To gain insight into the expression patterns of SufE2 and SufE3 we investigated the transcription abundance in different plant parts (Fig. 3). RNA blots indicated that SufE2 expression was high in flowers, while there was no detectable expression in roots, leaves, and stems (Fig. 3A). SufE2 transcript levels were also compared by RT-PCR for RNA isolated from root, leaf, stem, flower, and pollen (Fig. 3C). A very low transcript level was observed for root, leaf, and stem compared with flower and, especially, pollen. These results indicate that SufE2 expression is pollen-specific and are in agreement with publicly available microarray data. In Northern blots the SufE3 probe detected a band of comparable intensity for all major plant organs (Fig. 3B), suggesting that SufE3 mRNA is ubiquitously expressed.

**FIGURE 1. Schematic domain structure of Arabidopsis SufE1, SufE2, and SufE3.** TP, chloroplast targeting peptide.
The SufE Domains of SufE2 and SufE3 Stimulate CpNiFS Cysteine Desulfurase Activity—Since SufE1 was shown earlier to stimulate the cysteine desulfurase activity of CpNiFS, it was hypothesized that its homologues SufE2 and SufE3 have a similar stimulatory activity. To analyze this, the SufE domains were fused to a His6 tag and a thrombin cleavage site for efficient purification by immobilized metal ion chromatography. The His6 tags were cleaved off before use in cysteine desulfurase activity assays. The purified SufE domains of SufE2 and SufE3 were colorless and migrated as single bands on SDS-PAGE (Fig. 4, A and B). As expected, the SufE domains of SufE2 and SufE3 did not show any cysteine desulfurase activity by themselves (Fig. 4 C). CpNiFS cysteine desulfurase activity was barely detectable in the absence of SufE-like proteins. However, in the presence of equimolar amounts of the SufE2 domain, CpNiFS-mediated cysteine desulfurization increased ~40-fold. CpNiFS cysteine desulfurase activity increased 70-fold in the presence of the SufE domain of SufE3 when mixed in a 1:1 ratio. The stimulation of CpNiFS activity by the SufE domains was further increased when the SufE domain to CpNiFS ratio was 5:1 (Fig. 4 C).

An Intact SufE Domain Is Required in SufE3 for Complementation of an E. coli ΔNadA Strain—To test whether SufE3 represents an evolutionarily conserved protein and whether it encodes QS, a construct encoding the full-length SufE3 (pSufE3) was introduced and expressed in an E. coli mutant deficient in QS (ΔNadA). Furthermore, to test the requirement of the single cysteine residue in its SufE domain for QS activity, this was changed to serine (pSufE3C132S) and the modified plasmid was also introduced to E. coli ΔNadA. We compared the growth characteristics of ΔNadA transformed with pSufE3, pSufE3C132S, its own E. coli NadA homologue, or with an empty vector control. In the presence of nicotinic acid, a downstream metabolite of QS, all of the E. coli strains were able to grow on minimal media (Fig. 5 A). However, in the absence of NA, only the E. coli expressing intact SufE3 or the E. coli NadA homologue were able to grow. This ability to complement ΔNadA E. coli demonstrates that SufE3 is an evolutionarily conserved Arabidopsis protein with QS activity. The finding that SufE3C132S protein had lost its ability to complement ΔNadA (Fig. 5A), despite its accumulation to the same level as wild type SufE3 (Fig. 5B), indicates that the single Cys residue in the SufE

FIGURE 3. Expression analysis of SufE2 (A) and SufE3 (B) in different tissues. Total RNA from roots (R), leaves (L), stems (S), and flowers (F) was isolated. Ten microgram of total RNA was separated by electrophoresis, transferred to a Hybond-N membrane, and probed with 32P-labeled SufE2 and SufE3 cDNAs (upper panels) or 32P-labeled 18S rRNA probe (lower panels). C, RT-PCR of SufE2 in different tissues. Total RNA from roots (R), leaves (L), stems (S), and flowers (F) was used as a template for RT-PCR using SufE2-specific primers (SufE2) or actin2-specific primers (ACT2).

FIGURE 4. SufE2 and the SufE domain of SufE3 stimulate the cysteine desulfurase activity of CpNiFS. A and B, SDS-PAGE (14%) showing purified SufE2 (A) and the SufE domain of SufE3 (B). The gels were stained with Coomassie Brilliant Blue. Lanes A1 and B1, 5 μg of protein; lanes A2 and B2, 30 μg of protein; lanes A3 and B3, molecular mass standards. C, rate of cysteine desulfurase activity of CpNiFS, SufE2, and the SufE domain of SufE3, as well as combinations of SufE2 or the SufE domain of SufE3 with CpNiFS at two molar ratios (1:1 and 1:5). Data are the means ± S.E. of three experiments. Error bars smaller than the background activity are not shown.
domain of SufE3 is required for QS activity. The separately expressed NadA domain of SufE3 could not complement the E. coli ΔNadA mutant. However, this separately expressed NadA domain did not accumulate in the bacteria, suggesting that the SufE domain is required for expression in this system (not shown). Since the expression of the full-length SufE3 or the cysteine mutant in wildtype E. coli did not affect bacterial growth, there is no evidence for any dominant-negative effects.

SufE3 Is Essential for Normal Seed Development—To analyze the function of the SufE3 gene in Arabidopsis, we analyzed the phenotype of a T-DNA insertion mutant (SALK_075260) with a disruption in this gene. The single T-DNA in this line is inserted in the first exon of SufE3. Heterozygous SufE3 lines grown on soil showed the wild type phenotype. We analyzed and compared the seed development in a segregating SufE3 T-DNA insertion line to that of the wild type. In siliques of heterozygous SufE3 T-DNA insertion mutants one out of four seeds was aborted (Fig. 6, chi-squared test, $\chi^2 < 0.05$), while in the control plants 100% of the seeds were normal. We could not identify any plants that were homozygous for the SufE3 disruption in the T3 and T4 generation, further suggesting that the aborted siliques were due to loss of SufE3 and that SufE3 function is essential.

Expression, Purification, and Spectroscopic Characterization of the SufE3 Protein—Pure Arabidopsis SufE3, with a histidine tag at the C terminus, was first obtained by immobilized metal ion chromatography performed under aerobic conditions (outside of the glove box). Analysis by SDS-PAGE indicated an apparent molecular mass of 72 kDa. Gel filtration chromatography on Superdex-200 was used to determine the oligomerization state of SufE3. SufE3 protein eluted in a major peak corresponding to a dimeric form (140 kDa), but a minor amount of monomeric form (72 kDa) could also be observed. Combining the two Superdex fractions containing SufE3, 25 mg of pure SufE3 could be obtained from 800 mg of extract, in a more than 95% pure form as judged from SDS-PAGE (data not shown). The protein was slightly pink, suggesting the presence of an Fe-S metal center. However, the measured amount of iron and sulfide was substoichiometric with regard to the protein (0.15–0.2 iron and sulfide/monomer), showing that the protein isolated in an aerobic atmosphere was mainly in the apoprotein form.

Reasoning that the low cluster content was due to the aerobic manipulation of the protein and the oxygen-dependent degradation of the cluster, as already observed for the corresponding E. coli enzyme, SufE3 was purified under strictly anaerobic conditions inside a glove box. Under such conditions, a single protein to better than 95% purity was obtained as judged by SDS-PAGE (see inset in Fig. 7). The anaerobically purified protein was brown in color and its UV-visible spectrum, shown in Fig. 7, is characteristic for a (4Fe-4S)$_2$$^+$ cluster rather than 2Fe-2S, with only one absorption band in the visible range at 420 nm. Upon exposure to air, SufE3 bleached and the UV-visible spectrum changed with a decrease of the 420 nm absorption band ($t_{1/2} = 30$ min; data not shown). To verify the presence of a Fe-S cluster and to accurately determine its nature and stoichiometry, anaerobically purified protein was characterized using both Mössbauer and EPR spectroscopy and was chemically analyzed for its iron and sulfide content. As expected for a holoprotein with a 4Fe-4S cluster, the iron and sulfide determination revealed close to stoichiometric amounts of iron and sulfide, with 3.4 iron and 2.7 sulfide atoms/monomer. Fig. 8A shows the
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Mössbauer spectrum of a 250 μM SufE3 preparation obtained after expression in 57Fe-enriched M9 medium and anaerobic purification recorded at 78 and 4.2 K. This preparation contained 3 iron and 2.8 sulfide per polypeptide chain. At both temperatures the spectra are characterized by a doublet with isomer shift, δ = 0.45 (2) mm/s and quadrupole splitting, ΔEq = 1.12 (4) mm/s. At 78 K this species accounts for 85% of total iron. These parameters are consistent with a (4Fe-4S)2+ cluster. Similar parameters were obtained for E. coli NadA (42, 43). The spectrum at 78 K shows an additional minority doublet (15% of total iron) with parameters δ = 0.36 mm/s and ΔEq = 0.44 mm/s (dotted line), which is absent in the 4.2 K spectrum. Probably at 4.2 K this doublet is converted to a magnetically split spectrum expanding a large velocity region and under these conditions the spectral details cannot be resolved. The nature of these species is difficult to be identified, and presently it is attributed to paramagnetic species.

EPR spectra from this sample indicate the presence of weak signals at g = 4.3 and g = 2.0 corresponding to free ferric iron and (3Fe-4S)−1 species accounting for only 1–5% of total iron. Reduction of this sample with 2 mM dithionite under anaerobic conditions led to a rapid bleaching of the solution with disappearance of the 420 nm band (Fig. 7). During this reaction we obtained a complex EPR signal, which was analyzed as the superposition of two S = 1/2 species (Fig. 8B). One system (1) was characterized by g values at g = 2.07, 1.94, and 1.83, the second one (2), less rhombic, by g values at 2.03 and 1.94 (Fig. 8B). Temperature dependence and microwave power saturation properties of the two species are similar and in agreement with (4Fe-4S)2+ centers (data not shown). These two systems reflect a different environment for (4Fe-4S) clusters. The whole signal integrated to 80% of total iron. Upon exposure of the tube to air for 10 min, the EPR signal totally disappeared.

The SufE Domain Is a Sulfur Shuttle for Fe-S Formation in the NadA Domain of SufE3—To check whether there is a functional link between the SufE and NadA domains of SufE3, we investigated the ability of SufE3 to assemble an Fe-S cluster in its NadA domain in the presence of the purified chloroplast cysteine desulfurase CpNifS, cysteine and iron, and in the absence of exogeneous SufE. SufE3 was incubated with a catalytic amount of CpNifS, in the presence of 3 mM cysteine and a 10-fold molar excess of ferrous iron and the reaction monitored by UV-visible spectroscopy. After 5-min reaction time the solution became brown with a concomitant increase in the absorbance at 420 nm. After 1 h, there was no more change in absorbance. At this point the protein was treated with 1 mM EDTA for 30 min to remove unreactive iron and sulfide and desalted on a Nap10 column. The eluted protein was brown in color and displayed a UV-visible spectrum similar to that of the anaerobically purified holoprotein (data not shown). Iron and sulfide content analysis revealed the presence of 4.2 iron and 3.5 sulfide atoms/monomer in this reconstituted sample. When the same reaction was performed with either the truncated SufE3 protein lacking the NadA domain or with CpNifS539S (in which the reactive cysteine of the cysteine desulfurase has been changed to a serine residue), no increase in absorbance at 420 nm was observed even after overnight reaction, and no protein-bound iron was detected showing that no Fe-S was formed. These results and the E. coli complementation analysis (Fig. 5) together indicate that (i) the Fe-S cluster is localized in the NadA domain of SufE3, (ii) the cysteine desulfurase activity of CpNifS is required to provide the sulfur for this cluster, and (iii) the SufE domain of SufE3, via the conserved Cys residue, is essential for Fe-S cluster formation on the NadA domain of the same protein.

The Fe-S Cluster of SufE3 Is Absolutely Required for Activity—The ability of both anaerobically isolated and reconstituted SufE3 proteins (holo-SufE3 proteins) to catalyze formation of quinolinate was assayed. Iminoaspartate, a required but
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FIGURE 9. A, time curve of quinolinate formation. The assay was performed in 50 mM Na-HEPES, 100 mM KCl, pH 7.5, 5 mM DHAP, 33 mM L-aspartate, 25 mM fumarate, 15 μM of Fe-S-containing SufE3 protein, and 1.5 μM FAD-containing NadB at 37 °C. The amount of QA was determined as described under "Experimental Procedures." B, initial quinolinate formation as a function of DHAP concentration. The assay was performed as described above for 15 min. The amount of QA was determined as described under "Experimental Procedures." The line represents the fit generated with Kaleidagraph based on the equation \( V = V_{\text{max}}[S]/(K_m + [S]) \).

unstable substrate of quinolinate synthase (\( t_{1/2} \) of 2.5 min at 37 °C and pH 8), was generated from L-aspartate by the action of the NadB protein using fumarate as the electron acceptor. Under these conditions (described under "Experimental Procedures," in the presence of a saturating concentration of DHAP at 37 °C and pH 7.5, the holo-SufE3 enzyme catalyzed the formation of quinolinic acid with a specific activity of 0.050 μmol/min/mg in an assay that was linear over 35 min (Fig. 9A). In comparison, E. coli NadA displays a specific activity in the range of 0.015–0.026 μmol/min/mg (43).\footnote{S. Ollagnier de Choudens and M. Fontecave, unpublished results.}

Further experiments demonstrated that the reaction is protein-dependent and, interestingly, that it absolutely requires the Fe-S cluster of SufE3. Indeed, no production of quinolinic acid was observed after a 60-min reaction when apo-SufE3 was used as an enzyme or when the SufE domain of SufE3 (lacking the NadA domain) was used instead (data not shown). Furthermore, when SufE3 was air-exposed for 30 min, the rate of quinoline acid production dropped to 10% of the rate observed for the anaerobically maintained holoenzyme (data not shown). As shown in Fig. 9B, the initial reaction displayed a saturation behavior with respect to DHAP concentration under conditions in which L-aspartate is saturating. Under these conditions, a \( V_{\text{max}} \) value of 8.9 nmol/min and a \( K_m \) value of 1.1 mM were determined for DHAP. For comparison, the E. coli holo-protein NadA showed a \( V_{\text{max}} \) of 1.6 nmol/min and a \( K_m \) of 0.58 mM.\footnote{S. Ollagnier de Choudens and M. Fontecave, unpublished results.}

\section*{DISCUSSION}

In this study, we characterize two novel SufE-like proteins from Arabidopsis, which we call SufE2 and SufE3. All three SufE-like Arabidopsis proteins stimulate the cysteine desulfurase activity of chloroplastic CpNifS. SufE1 was found earlier to be expressed in all tissues, with a 2-fold higher expression in leaves compared with non-green tissues (37). The elevated level of SufE1 expression in leaves is typical for proteins involved in photosynthesis. SufE2 expression is almost undetectable in all organs tested except for flowers and particularly pollen. Interestingly, micro-array analysis indicates that, while CpNifS is expressed in pollen, SufE1 expression in pollen is very low (Genesearcher). The available data indicate that SufE2 may have a specific but still unidentified function in Fe-S assembly in the plastids of pollen. Alternatively, it may serve in the sulfuration of other metabolites. Very little is known about the proteome of pollen plastids and to our knowledge Fe-S proteins with a function in this compartment have not been identified yet. A class of plastids known as elaioplasts are proposed to have a function in the storage and metabolism of oils proposed to be important in pollen wall maturation (54).

The SufE3 mRNA was ubiquitously expressed, but in contrast to the abundant SufE1, the SufE3 mRNA was barely detectable. Interestingly, mature SufE3 consists of both a SufE-like domain and a domain similar to the bacterial quinolinate synthase NadA. Indeed, SufE3 displays both a SufE activity and a quinolinate synthase activity. The results presented here provide the following evidence that SufE3 is the A. thaliana quinolinate synthase: (i) its homology with NadA from E. coli is strong; (ii) SufE3 contains a highly oxygen-sensitive (4Fe-4S)\(^2+\) cluster, characterized by UV-visible, EPR and Mössbauer spectroscopy, very similar to that in NadA from E. coli (42, 43); (iii) SufE3 displays a quinolinate synthase activity absolutely dependent on the presence of the cluster; (iv) SufE3 complements a \( \Delta \)NadA E. coli mutant strain. Interestingly, this complementation requires the intact SufE domain of SufE3. The 4Fe-4S cluster of SufE3 can be reconstituted by its own SufE domain in the presence of CpNifS, cysteine and ferrous iron. Knock-out of SufE3 was embryo-lethal. We conclude that SufE3 is the quinolinate synthase enzyme of Arabidopsis, involved in a critical step during NAD biosynthesis.

Since plants have evolved three activators of sulfur mobilization by CpNifS, it might be expected that some of these can complement one another. Still, the embryo-lethal phenotypes of SufE1 and SufE3 knockouts indicate that they cannot. Obvi-
ously, SufE1 cannot complement SufE3 because it has no quinolinate synthase activity. SufE2 cannot complement loss of SufE1 function in vegetative tissues because it is only expressed in pollen. Several explanations may be given for the lethal phenotype of the SufE1 mutant in the presence of functional SufE3, which has a similar expression pattern as SufE1. The SufE3 expression level may be too low to compensate for loss of SufE1. Also, SufE1 localizes to both mitochondria and chloroplasts (36), while SufE3, as far as is clear from studies so far, is only present in the plastids. However, we propose that the most important reason for the lack of SufE3 complementation of a SufE1 mutant is that the SufE activity of SufE3 is likely dedicated to Fe-S cluster formation in its own quinolinate synthase domain.

It is tempting to suggest that the fusion of a SufE domain and a NadA domain in a single protein in SufE3 has a functional significance. We propose that in A. thaliana the SufE3/NadA protein contains a SufE domain to specifically shuttle sulfur atoms from CpNiFS to the NadA domain, for the assembly of the catalytically essential 4Fe-4S cluster, through an intraproteic sulfur transfer from the cysteine 132 to the active site of the NadA domain. Accordingly, the plasmid allowing expression of the SufE3 mutant, in which cysteine 132 was changed to a serine, could not complement an E. coli ΔNadA mutant strain. It is interesting to note that also in E. coli NadA maturation/activation seems not to use the general Fe-S cluster biosynthetic ISC pathway. In E. coli, there is no fusion with a SufE domain to control sulfur transfer, but instead a specific CsdA/CsdE cysteine desulfurase system is utilized (48). This is intriguing but might be a consequence of the importance of the NAD cofactor, which requires a highly dedicated biosynthetic machinery. In the oxygenic chloroplast this is likely even more important, since SufE3 is highly O2-sensitive. In a light-exposed chloroplast the oxygen levels are supersaturated, and the purified protein already lost its activity within 30 min upon exposure to atmospheric oxygen levels. Likely the SufE domain of SufE3 has a continuous role in repairing/reconstituting the Fe-S cluster in the NadA domain of the same protein, to maintain protein function. Thus, the reason why this particular protein needs its own SufE is likely its extreme sensitivity to oxygen, combined with its intracellular location in an oxygenic organelle.

The previously characterized SufE1 contains a BolA domain at its C-terminus (36, 37). Through bioinformatic analysis it was suggested that BolA-like proteins may interact with monothiol glutaredoxins (55), proteins with proposed regulatory roles that are represented by an extensive family in plants (56). Since plastids are the main site of cysteine synthesis in plants, there should be a mechanism to regulate CpNiFS cysteine desulfurase activity and prevent depletion of the cysteine pool. We think it is possible that the BolA domain of SufE1 plays a role in the regulation of cysteine desulfurase activity in response to the need for Fe-S cluster biosynthesis and repair. Such a BolA domain is not present in SufE2, SufE3, or bacterial SufE proteins. In bacteria the expression of key enzymes in cysteine synthesis can be regulated so as to maintain the cysteine pool in response to the demand for Fe-S proteins. In Arabidopsis, SufE2 perhaps would not require tight regulation because of its presence only in pollen, which is not likely a major site of cysteine synthesis. The isolated SufE domain of SufE3 increased CpNiFS cysteine desulfurase activity 70-fold, so to a larger extent than SufE2 and SufE1, indicating efficient interaction with CpNiFS. Because NAD synthesis is likely to be very crucial, yet at the same time the Fe-S center of SufE3 is extremely sensitive to oxidative damage, it may be desirable for SufE3 always to have access to CpNiFS. SufE3 may therefore be regulated only through the status of the Fe-S cluster in its NadA domain. The detectable but low mRNA expression levels suggest that in the plastids SufE3 is present at much lower levels than SufE1 and CpNiFS. Therefore, SufE3 binding to CpNiFS would not significantly affect SufE1-CpNiFS interactions.

The essentiality of CpNiFS for Fe-S cluster assembly in chloroplasts has been demonstrated both in vitro (24) and in vivo (57). Silencing of CpNiFS was shown recently to impede photosynthetic electron transport, nitrogen and sulfur assimilation, and carbon fixation (57). The data presented here indicate an additional role of the cysteine desulfurase CpNiFS in NAD synthesis.

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