The *AtNFS2* gene from *Arabidopsis thaliana* encodes a NifS-like plastidial cysteine desulphurase

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NifS-like proteins are cysteine desulphurases required for the mobilization of sulphur from cysteine. They are present in all organisms, where they are involved in iron–sulphur (Fe–S) cluster biosynthesis. In eukaryotes, these enzymes are present in mitochondria, which are the major site for Fe–S cluster assembly. The genome of the model plant *Arabidopsis thaliana* contains two putative NifS-like proteins. A cDNA corresponding to one of them was cloned by reverse-transcription PCR, and named *AtNFS2*. The corresponding transcript is expressed in many plant tissues. It encodes a protein highly related (75 % similarity) to the *slr0077*-gene product from *Synechocystis* PCC 6803, and is predicted to be targeted to plastids. Indeed, a chimaeric

AtNFS2–GFP fusion protein, containing one-third of *AtNFS2* from its N-terminal end, was addressed to chloroplasts. Overproduction in *Escherichia coli* and purification of recombinant *AtNFS2* protein enabled one to demonstrate that it bears a pyridoxal 5'-phosphate-dependent cysteine desulphurase activity *in vitro*, thus being the first NifS homologue characterized to date in plants. The putative physiological functions of this gene are discussed, including the attractive hypothesis of a possible role in Fe–S cluster assembly in plastids.

Key words: desulfurase, iron–sulphur cluster, plastid, thale cress, thiamin.

INTRODUCTION

Pyridoxal 5'-phosphate (PLP)-dependent cysteine desulphurases are essential enzymes required in all living organisms for the synthesis of iron–sulphur (Fe–S) clusters. They catalyse the formation of L-alanine and elemental sulphur by using L-cysteine as a substrate. This activity was first described for the NifS protein from *Azotobacter vinelandii* which is involved in nitrogenase Fe–S cluster formation [1]. NifS-like proteins, called IscS, were later observed in *A. vinelandii* [2], as well as in a number of other organisms such as *Escherichia coli*, where IscS is involved in Fe–S cluster biosynthesis [3]. The use of yeast as a eukaryotic model recently demonstrated that at least ten mitochondrial proteins participate in Fe–S cluster biogenesis [4]. Among the genes encoding these proteins, mutation of the *NFS1* gene, the *Saccharomyces cerevisiae* NifS-like gene, caused a decrease in mitochondrial Fe–S protein activities, such as those of aconitase and succinate dehydrogenase [5,6]. In human cells, in addition to its mitochondrial location, IscS has also been shown to be located in the cytoplasm [7], although mitochondria play a crucial role in Fe–S cluster formation of extramitochondrial Fe–S proteins [4,8].

In addition to their cysteine desulphurase activity, some NifS-like proteins are able to catalyse the removal of the selenium atom from L-selenocysteine when L-selenocysteine is used as a substrate instead of L-cysteine [9]. Furthermore, besides their major role in Fe–S cluster formation, NifS-like proteins are involved in unrelated functions through their sulphur transferase activity. These additional functions include participation in tRNA splicing in yeast [10] or biosynthesis of thiamin, 4-thiouridine and NAD⁺ in *E. coli* [11] and biosynthesis of molybdopterin [12] (see [13] for a review).

In plants, the complete sequence of the *Arabidopsis thaliana* (thale cress) genome [14] allowed one to look for open reading frames (ORFs) whose corresponding proteins share sequence similarities to NifS proteins. Two candidate genes have been identified [15,16]. The putative protein encoded by one of these genes (*AtNFS1*) is 70 % identical with Slr0387, one of the three NifS-like proteins from *Synechocystis* PCC 6803, which is the most similar to NifS from *A. vinelandii*. It has recently been proposed to be located in mitochondria, on the basis of the intracellular localization of a fusion protein between the N-terminal end of the *AtNFS1* protein and green fluorescent protein (GFP) [17]. The other *Arabidopsis* putative NifS protein, *AtNFS2*, is predicted to be targeted to plastids, raising the possibility that these organelles, in addition to mitochondria, may be also able to synthesize Fe–S clusters in plant cells [15,16]. Indeed, the assembly of a ferredoxin Fe–S cluster has been shown to occur in isolated chloroplasts [18], in a cysteine-, NADPH- and ATP-dependent reaction [19], but no enzyme involved in this assembly process has been identified so far.

In the present study we have cloned the cDNA corresponding to the putative *A. thaliana* NifS protein predicted to be targeted to plastids; it revealed annotation errors of the corresponding gene that we have corrected. This gene is expressed in many plant tissues. The recombinant protein was overexpressed in *E. coli* and purified, allowing us to show its ability to catalyse L-alanine formation *in vitro* using L-cysteine as a substrate. Finally, fusion of the sequence encoding the N-terminal part of this protein with the GFP coding sequence led to the observation that this chimaeric protein is targeted to plastids of *A. thaliana* cells. These data strongly suggest that plastids could be a site for Fe–S cluster formation in plant cells, and this point is discussed.

Abbreviations used: CHX, cycloheximide; DTT, dithiothreitol; Fe–S, iron–sulphur; GFP, green fluorescent protein; NLS, nuclear localization sequence; NTA, nitrilotriacetate; OPA, o-phthalaldehyde; ORF, open reading frame; PEG, poly(ethylene glycol); PLP, pyridoxal 5'-phosphate; RT-PCR, reverse-transcription PCR.

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The *AtNFS2* cDNA sequence has been deposited in the GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession number AY078068.

EXPERIMENTAL

Plant material

A. thaliana (ecotype Columbia) was grown in a greenhouse (23 °C, light intensity maintained above 300 $\mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$, 16 h/8 h day/night period) on Humin-Substrat N2 Neuhaus (Klasmann-Deilmann, Geeste, Germany) for 35 days. Samples were harvested, frozen in liquid nitrogen, and stored at -80°C prior to RNA extraction.

Cell suspensions of *A. thaliana* (ecotype Columbia, strain T87) were cultured as previously described [20].

Reverse-transcription (RT-PCR), cloning and analysis of AtNFS2 cDNA

Total RNA was prepared by the guanidine procedure [21] from various tissues of *A. thaliana* plants. cDNAs were synthesized using Moloney-murine-leukaemia-virus reverse transcriptase RNase H(−) point mutant (Promega Corp., Madison, WI, U.S.A.) as described by the supplier, using 5 μg of total RNA as template. One-fiftieth of the reverse-transcription reaction mixture was used as a template for 30 cycles of PCR in order to amplify AtNFS2 cDNA. The primers used were 5′-TCCGTCTAGACTCACTCTTGTTCATTTCGTCTTCTCCACC-3′ and 5′-TCCGGGTACCCCACTCCCATTGATCATATAAAGCA-GATTT-3′ (restriction sites indicated in **bold** type), designed to clone the PCR product at *Xba*I–*Kpn*I sites of pBKS+ vector (Stratagene, La Jolla, CA, U.S.A.) and sequenced. Alignment was performed by using the PIMA v.1.4 algorithm [22]. A phylogenetic tree was calculated by the Neighbour-Joining method using ClustalX v. 1.8 [23] and then visualized with TreeView v. 1.6.6.

Overexpression and purification of hexahistidine-tagged AtNFS2 (AtNFS2-His₆)

A portion of the AtNFS2 cDNA encoding amino acids 35–464 was amplified by PCR using primers 5′-TCCGCATATGGCCGCTTCCTCCGCCACCAT-3′ and 5′-TCCGCTCGAGTTTGAAAGAGTTGAAGAAGCTCACAGT-3′, enabling one to clone the product at *Nde*I and *Xho*I sites of pET20b+ vector (Novagen Inc., Madison, WI, U.S.A.). *E. coli* strain BL-21 was used as a host for overexpression of the His-tagged AtNFS2 protein. Crude extracts were obtained by sonicating an overnight culture in an extraction buffer containing 150 mM NaCl and 10 mM imidazole in 50 mM NaH_2PO_4 buffer, pH 8.0. For purification of AtNFS2 protein in a soluble form, a 1-litre culture of pET20b:AtNFS2-His₆-transformed bacteria was incubated overnight at 30 °C in Luria–Bertani medium containing 1 % glucose (w/v). After centrifugation, bacteria were therefore resuspended in 0.8 litres of Luria–Bertani medium without glucose to allow rapid expression of the protein before harvest and left with shaking at 30 °C for 2 h. Crude extracts were obtained as described above in 50 ml of extraction buffer, and centrifuged for 30 min at 10000 *g*. The supernatant, containing soluble proteins, was incubated with 1 ml of Ni-nitrilotriacetate (NTA)-agarose resin (Qiagen Inc., Valencia, CA, U.S.A.) at 4 °C for 1 h, and then loaded on a liquid-chromatography column (0.7 cm $\times$ 10 cm; Sigma). The resin was washed in the extraction buffer containing 20 mM imidazole, and the adsorbed proteins were then eluted with the same buffer containing 250 mM imidazole (elution buffer).

Proteins were separated by SDS/13 %-(w/v)-PAGE [24] and stained with Brilliant Colloidal Blue-G (Sigma).

Protein concentration was measured with the Bio-Rad (Hercules, CA, U.S.A.) protein assay kit, using BSA as a standard.

UV/visible spectra were obtained on a Uvikon 930 spectrophotometer (Kontron Instruments, Watford, U.K.) using a protein concentration of 2 $\text{mg} \cdot \text{ml}^{-1}$ in elution buffer.

Cysteine desulphurase assay

Cysteine desulphurase activity was assayed by measuring the formation of alanine from cysteine added to the reaction medium. Raw extracts were dialysed overnight at 4 °C in dialysis cassettes (Slide-A-Lyzer 10K; Pierce, Rockford, IL, U.S.A.) in extraction buffer. Samples were incubated for various times (15–60 min) at 25 °C in 20 mM Hepes/NaOH, pH 8.5, containing 0.5 mM cysteine (Sigma) and 10 μM dithiothreitol (DTT). The reaction (final volume 500 μl) was stopped by the addition of 500 μl of 100 % ethanol, and immediately stored at -20°C prior to analysis. Alanine was detected by HPLC after derivatization with *o*-phthalaldehyde (OPA) reagent [5 $\text{g} \cdot \text{litre}^{-1}$ OPA, 0.4 % (v/v) β -mercaptoethanol/10 % (v/v) methanol/0.4 M sodium borate buffer, pH 9.5] and with iodoacetic reagent [0.5 % (w/v) iodoacetic acid/0.4 % (w/v) boric acid, adjusted to pH 9.5 with NaOH]. A 70 μl portion of each reagent was added to 20 μl of sample, and 50 μl were injected into the HPLC column (Spherisorb ODS-2; 150 mm $\times$ 4.6 mm) 1 min after the reaction. Derivatized amino acids were then eluted by a binary gradient [A: 50 mM sodium acetate (pH 5.7)/tetrahydrofuran (3 %, v/v); B: 5 % (v/v) tetrahydrofuran in methanol]. The A/B ratio ranged linearly from 90:10 (injection) to 37:63 (19 min) and then 0:100 for 1 min. Alanine retention time was checked at regular intervals by using samples in which cysteine was replaced by alanine.

Subcellular localization of AtNFS2–GFP fusion protein

A portion of AtNFS2 cDNA encoding amino acids 1–154 was amplified by PCR using the primers (5′-TCCGTCTAGACTCACTCTTGTTCATTCGTCTTCTCCACC-3′ and 5′-TCCGCGGATCCCCCATGAATAAGCTACAAGATTGATGGC-3′), which allowed cloning of the PCR product at *Xba*I–*Bam*HI sites of a modified pBI221 vector (Clontech, Palo Alto, CA, U.S.A.). This vector contains a soluble modified red-shifted GFP ('SmRS–GFP') [25] at *Bam*HI–*Sac*I sites.

For protoplasts transformation, 50 ml of a 6-day-old cell culture of *A. thaliana* (ecotype Columbia, strain T87) were centrifuged for 5 min at 75 *g*. Cells were resuspended in 25 ml of a buffer containing 1 % (w/v) cellulase Onozuka R-10, 0.25 % macerozyme R-10 (Duchefa, Haarlem, The Netherlands), 8 mM CaCl_2 , 0.5 M mannitol, 5 mM Mes, pH 5.5, and incubated for 5 h at room temperature. Protoplasts were harvested by centrifugation for 5 min at 75 *g* and washed twice in 12 ml of W5 wash solution (154 mM NaCl/125 mM CaCl_2 /5 mM KCl/5 mM glucose/0.5 M mannitol, adjusted to pH 5.8 with KOH). The pellet was first resuspended in 12 ml of mannitol/Mg solution (15 mM MgCl_2 /0.4 M mannitol/0.1 % Mes, pH 5.6), centrifuged for 5 min at 75 *g*, resuspended in approx. 2.5 ml of mannitol/Mg solution and left for 30 min on ice. Protoplasts were counted using a haemocytometer and their concentration was adjusted to 9×10^6 cells $\cdot \text{ml}^{-1}$ with mannitol/Mg solution. A 50 μg portion of plasmid DNA, 100 μg of salmon sperm DNA and 300 μl of poly(ethylene glycol) (PEG) solution [20 % PEG 6000/0.4 M mannitol/0.1 M $\text{Ca}(\text{NO}_3)_2$, adjusted to pH 8.0 with KOH] were added to 300 μl of the protoplast solution, very gently mixed and left for 30 min at room temperature. The solution was

Figure 1 Comparison of the amino acid sequences of NifS cysteine desulphurase proteins

(A) *AtNFS2*-gene organization. Empty boxes represent exons, and black triangles represent introns, for which the length is indicated. (B) Primary sequence alignment of AtNFS2 with other proteins. The deduced amino acid sequence of AtNFS2 cDNA is compared with those of the predicted AtNFS1 protein (GenBank® accession number O49543), yeast NFS1p (P25374), *E. coli* IscS (P39171), and *Synechocystis* Csd3 (Q55793). Amino acid residues conserved in most sequences are boxed in black, and those which are partially conserved (identical or similar) are boxed in grey. Alignment was performed by using the PIMA v.1.4 algorithm [22], modified at the C-terminal to enhance homology to show the conserved cysteine residues. Asterisks denote amino acids which may be involved in catalysis ([29]; see the text for details). The potential catalytic cysteine residue and potential PLP-binding lysine residue are indicated with a black square and a black circle respectively. (C) Phylogenetic tree showing the relationships between the five sequences shown in (B). Alignment was performed with PIMA v. 1.4 [22], the tree was subsequently calculated by the Neighbour-Joining method using ClustalX v. 1.8 [23] and then visualized with TreeView v. 1.6.6.

very slowly diluted with 10 ml of W5 solution, then pelleted by centrifugation for 5 min at 75 *g*. Protoplasts were further resuspended in 3 ml of protoplast culture medium composed of the *Arabidopsis* cell-culture medium plus 0.4 M mannitol and 0.4 M glucose, and left at 23 °C for 24 h under continuous light (100 $\mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$). Green fluorescence, chlorophyll red autofluorescence and transmission images were monitored using a confocal laser scanning microscope (MRC 1024; Bio-Rad) with Kr/Ar laser excitation (488 nm). The fluorescence signals were detected at 530 ± 16 nm for GFP and 660 ± 16 nm for chlorophyll autofluorescence.

RESULTS

Cloning of AtNFS2 cDNA, originating from an *A. thaliana* NifS-related gene

A BLAST search [26] on the *A. thaliana* translated genome (www.arabidopsis.org) using the protein sequence encoded by

ORF *slr0077* of *Synechocystis* (sometimes referred to as *SyCsd3* [27]; accession number Q55793) revealed one putative protein (accession number AAF22900), already annotated as a putative NifS. This database mining output was in perfect agreement with indications reported very recently by others [15,16]. This predicted protein showed 57 % identity and 68 % homology with *SyCsd3* over 449 amino acids. The corresponding gene (*At1g08490*, BAC T27G7.17) is located on chromosome 1. In order to confirm these *in silico* (in or by means of computer simulation) searches, we cloned the corresponding cDNA by RT-PCR using *A. thaliana* leaf RNA. Sequencing and analysis of this cDNA (accession number AY078068) indicated that it contains a 1392 bp ORF encoding a 464-amino-acid protein for a predicted molecular mass of 50485 Da. Differences in amino acid sequence in the central and C-terminal regions between the translated ORF sequence and the database protein sequence were observed. This was due to important annotation errors of the corresponding gene, including wrong 5'-splicing site for

Figure 2 Non-quantitative RT-PCR detection of *AtNFS2* mRNA in various tissues of *Arabidopsis*

Total RNA was extracted from flowers (Fl), siliques (Si), floral stalk (St), leaves (L) and roots (R) of 35-day-old *Arabidopsis* plants. Specific *AtNFS2* primers were used to amplify a 1291 bp sequence of the cDNA. Two primers were also used to amplify a 643 bp fragment of *Actin-2* cDNA as a positive control of RNA extraction and reverse transcription.

intron 4, and introns 3, 7 and 8 not being recognized as such. Comparison of the cloned cDNA with the genomic sequence (T27G7.17) indicated that this gene is actually composed of nine exons and eight introns (Figure 1A). Kushnir et al. [17] already identified an unrelated *Arabidopsis* NifS gene (F6H11.150), likely to encode a mitochondrial protein annotated as *AtNFS1* (accession number O49543). The gene we are describing in the present work is therefore referred to as *AtNFS2*.

Alignment of the cDNA-encoded *AtNFS2* protein with *Synechocystis* SyCsd3 showed that they share 63 % identity and 75 % homology over a 409-amino-acid sequence, excluding the first 40 N-terminal amino acids (Figure 1B). This N-terminal peptide shares all the characteristics of a transit peptide found in precursor proteins targeted to the plastids within plant cells [28]. This observation is in agreement with the observations reported by Seidler et al. [15]. Unlike *AtNFS1* protein, which is close in primary sequence to other NifS proteins found in metazoa or protists (group I), phylogenetic analysis showed that *AtNFS2* and SyCsd3 predicted proteins belong to group II, which consists of the most divergent NifS proteins ([16]; Figure 1C).

Nevertheless, *AtNFS2* protein possesses all the conserved residues playing important roles in NifS function, as proposed by Kaiser et al. [29] (Figure 1B). This includes Cys⁴¹⁸ involved in the binding of the substrate, three residues involved in anchoring the cysteine with a salt bridge and hydrogen bond (Arg⁴³³, Asn¹⁰² and Asn²²⁴), His¹⁷² involved in initial deprotonation of the substrate, Lys²⁷⁵ which is the PLP cofactor-binding site, Asp²⁴⁹ and Gln²⁵², which bind the pyridine nitrogen and the phenolate oxygen of PLP, and other residues involved in anchoring the phosphate group (Thr¹⁴⁴, His²⁷⁴, Thr²⁷² and Thr³²⁶). However, a 25-amino-acid conserved sequence in the C-terminus and present in other cysteine desulphurases such as *E. coli* IscS, yeast NFS1p and *Arabidopsis* *AtNFS1* is absent both in *AtNFS2* and *Synechocystis* SyCsd3 predicted gene products.

Expression of *AtNFS2* in various organs of *Arabidopsis*

Non-quantitative RT-PCR experiments were performed on total RNA extracted from various organs of 35-day-old *Arabidopsis* plants cultured under standard conditions. A PCR product of the expected size was well observed in each organ tested [flowers, siliques (pods), floral stalks, leaves and roots], after 30 cycles of amplification (Figure 2). This indicates that the *AtNFS2* gene is widely expressed throughout the plant and that its expression is not restricted to a particular organ.

Subcellular localization of *AtNFS2* protein

The high homology and close phylogenetic relationship between *AtNFS2* and *Synechocystis* Csd3 ([16]; Figure 1C) led to the idea

Figure 3 Subcellular localization of *AtNFS2*–GFP fusion protein

Arabidopsis cell suspension culture protoplasts were transformed using a control vector (35S–GFP) (A–C) and *AtNFS2*–GFP construct (D–F) encoding an in-frame fusion protein between N-terminal amino acids 1–154 of *AtNFS2* protein and the GFP. After 24 h of transient expression, cells were observed using a confocal laser scanning microscope (MRC1024; Bio-Rad) and a 60 × Plan-Apo oil-immersion objective. Confocal view of the cell (A and D), chlorophyll red autofluorescence used as a chloroplast marker (B and E) and green fluorescent signal (C and F) are shown. The scale bar corresponds to 10 µm.

that *AtNFS2* could be a plastidial protein, as cyanobacteria and plant chloroplasts share a common ancestor [30]. Moreover, as mentioned above, the N-terminal extension of *Arabidopsis* *AtNFS2* protein relative to *E. coli* IscS or *Synechocystis* Csd3 proteins possesses features of a plastidial transit peptide [28], and *AtNFS2* is predicted to be a chloroplast protein by the ChloroP software [31].

In order to confirm this prediction, the sequence encoding the N-terminal third of the protein (amino acids 1–154) was fused in-frame upstream of the GFP reporter gene, and transient expression of this fusion protein was monitored in *A. thaliana* protoplasts by confocal microscopy. As shown in Figures 3(E) and 3(F), chlorophyll autofluorescence co-localized with GFP fluorescence when fused to *AtNFS2*, whereas GFP fluorescence was spread throughout the cytoplasm and the nucleus when GFP alone was expressed as a control (Figures 3B and 3C). These results suggest that the *AtNFS2* N-terminal sequence is sufficient to address the chimaeric product into plastids, consistent with a plastidial localization of the *AtNFS2* protein.

Table 1 Cysteine desulphurase activity of crude extract of pET20b+ :AtNFS2-transformed *E. coli* cells and of AtNFS2-His₆-enriched fraction

Cysteine desulphurase activity was measured by HPLC monitoring of alanine formation, after 30 min of incubation at 25 °C (pH 8.5) in the presence of 0.5 mM L-cysteine using a protein concentration of 1 mg · ml⁻¹. Data from the crude extract represent the cysteine desulphurase activity of pET20b+ :AtNFS2-transformed cells minus that of pET20b+ -transformed cells in order to subtract the endogenous *E. coli* cysteine desulphurase activity. Results are means ± S.D. (*n* = 3) for a typical purification procedure.

	Quantity of protein (mg)	Specific activity (nmol of alanine · min ⁻¹ · mg ⁻¹)	Total activity (nmol of alanine · min ⁻¹)
Crude extract (pET20b+ :AtNFS2-His ₆)	229	0.033 ± 0.003	7.6 ± 0.8
AtNFS2-His ₆ fraction	5.3	0.807 ± 0.073	4.3 ± 0.4

Figure 4 Purification procedure of AtNFS2-His₆ and spectral characterization

(A) SDS/PAGE crude extract protein patterns of *E. coli* transformed by the empty vector (pET20b+) or by the vector containing AtNFS2 cDNA (AtNFS2-His₆). Protein patterns of the purification steps on Ni-NTA-agarose resin are shown: lane 1, insoluble proteins from the crude preparation; lane 2, soluble proteins incubated with the resin; lane 3, proteins eluted with 10 mM imidazole (flow-through); lane 4, column wash (20 mM imidazole); lane 5, eluted proteins (250 mM imidazole). All lanes were loaded with 30 µg of protein (except lane 5, loaded with 8 µg) and stained with Brilliant Colloidal Blue-G. Arrows indicate the AtNFS2-His₆ protein at the expected mass. (B) Absorption properties of the AtNFS2-His₆ preparation (lane 5 shown in A). Absorption optima were obtained at 418 nm, in the absence of cysteine (bold line) or at 425 nm, and 341 nm, in the presence of 0.5 mM cysteine (thin line). The protein concentration was 2 mg · ml⁻¹ in elution buffer, pH 8.0.

Overexpression and purification of AtNFS2 protein

E. coli was used as a host for overexpression of AtNFS2 protein. A portion of the AtNFS2 cDNA encoding amino acids 35–464 was cloned into the pET20b+ vector to overexpress a tagged AtNFS2-His₆ protein lacking most of the predictable transit peptide sequence. Comparison of protein patterns between crude extracts from pET20b+ and pET20b+ :AtNFS2 transformed bacteria pointed out the presence of an over-accumulated protein in the latter case, with an observed mass of 45 kDa, close to that

Figure 5 Cysteine desulphurase activity of the AtNFS2-His₆-enriched fraction

The quantity of alanine formed (nmol · mg⁻¹) at various times, from 0 to 60 min, is shown. The reaction was performed at 25 °C, at pH 8.5 and in presence of 0.5 mM cysteine as a substrate, using a protein concentration of the AtNFS2-His₆-enriched fraction (Figure 4A, lane 5) of 2 mg · ml⁻¹. The results are means ± S.D. (*n* = 3) for a typical purification procedure.

predicted for AtNFS2-His₆ (Figure 4A; crude extracts panel). Low solubility of the protein after a classical overnight culture at 37 °C (results not shown) was circumvented by growth of bacteria at 30 °C in presence of 1 % (w/v) glucose. This enabled one to obtain AtNFS2-His₆ as a soluble protein (Figure 4A, lane 2), and Ni-NTA-agarose resin-based purification raised a highly enriched preparation of this recombinant protein (Figure 4A, lane 5).

This purified fraction exhibited the characteristic yellow colour observed for other PLP-containing enzymes such as all NifS proteins studied to date. UV/visible spectra revealed an absorption optimum at 418 nm (Figure 4B), consistent with the presence of PLP associated with the protein moiety [1]. Moreover, we investigated whether cysteine, the potential substrate of this class of protein, induced a change in the spectral properties of AtNFS2-His₆, as reported for *A. vinelandii* NifS [1]. Addition of 0.5 mM cysteine in the protein sample actually induced a shift of the major peak to 425 nm, decreasing its intensity, and induced the appearance of a new peak at 341 nm, which only existed as a shoulder in absence of cysteine (Figure 4B). These spectral changes were not observed when 0.5 mM alanine was added instead of L-cysteine (results not shown).

L-Cysteine desulphurase activity of AtNFS2 protein

To assess whether AtNFS2 acts as an L-cysteine desulphurase *in vitro*, raw extracts and AtNFS2-His₆ fraction (Figure 4A, lane 5) were assayed for their ability to synthesize alanine from

cysteine, by mean of HPLC. AtNFS2-His₆ purification led to a 25-fold increase in specific activity in the enriched fraction as compared with that of the raw extract (Table 1). In the presence of 0.5 mM L-Cys, the amount of alanine formed varied linearly with the quantity of AtNFS2-His₆ protein added in the sample within 30 min (results not shown). No alanine production was observed when cysteine was omitted from the reaction medium (results not shown). This is the first evidence for a cysteine desulphurase activity harboured by the AtNFS2 protein.

Cysteine desulphurase activity of the AtNFS2-His₆-enriched fraction was also assayed at various times (0–60 min), using the same conditions (0.5 mM cysteine, 25 °C) and a protein concentration of 2 mg · ml⁻¹, as shown in Figure 5. The highest specific activity (slope) was observed within the first 15 min (1.05 nmol of alanine formed · min⁻¹ · mg⁻¹ of protein). Altogether, these observations indicate that AtNFS2-His₆ protein can indeed produce alanine from cysteine *in vitro*, and is therefore a functional L-cysteine desulphurase.

DISCUSSION

In the present paper we report the cloning of AtNFS2 cDNA encoding the first NifS cysteine desulphurase from *A. thaliana* characterized to date. The AtNFS2 primary sequence is closely related to that of the NifS-like protein encoded by ORF *slr0077* from *Synechocystis* PCC 6803 (SyCsd3).

The *Synechocystis* genome contains two other ORFs (*slr0387* = SyCsd1 and *slr0704* = SyCsd2) encoding NifS-like cysteine desulphurases, and one ORF (*slr2143*) encoding a C-DES [cyst(e)ine desulphurylase]. All of these, as well as a cysteine/cystine C-S-lyase [32], appear to facilitate Fe–S cluster assembly by measuring the *in vitro* formation of holoferridoxin from apoferredoxin [27,33] (see also [15]). However, the role of the *Synechocystis* AtNFS2-like protein remains unknown, although it seems that its corresponding ORF is essential for growth of this cyanobacterium (reported in [15]).

Our data are consistent with a plastidial subcellular localization of AtNFS2 (Figure 3), as opposed to the *AtNFS1* gene product, which appears to be targeted to mitochondria [17]. In eukaryotic cells, the importance of mitochondria in Fe–S cluster biogenesis has already been outlined [4], and the few data available suggest that it is also likely to be the case in plant cells [17]. AtNFS1 protein is therefore a good candidate to be involved in Fe–S cluster biogenesis in plant mitochondria. However, photosynthesis reactions require many Fe–S cluster-containing proteins, and the assembly of the Fe–S cluster of plant ferridoxin was shown to occur in isolated chloroplasts [18], although no enzyme involved in this assembly process has been identified so far. Counterparts of these proteins exist in cyanobacteria, for which only a little information has yet been published concerning the molecular basis of Fe–S cluster assembly [15,32–34]. As the chloroplast ancestor is phylogenetically related to cyanobacteria [30], it can be postulated that a conserved mechanism occurs in cyanobacteria and chloroplast concerning Fe–S cluster synthesis within this organelle, as it has already been reported for other pathways, such as isoprenoid biosynthesis for instance [35].

In bacteria such as *E. coli*, IscS acts together with IscU for *de novo* biosynthesis of Fe–S clusters [36]. No gene encoding IscU-like proteins is present in the fully sequenced *Synechocystis* PCC 6803 genome, although this class of scaffold proteins is highly conserved throughout evolution [37]. A 25-amino-acid C-terminal sequence of the *E. coli* IscS protein has recently been shown to be important for IscS/IscU interaction occurring in Fe–S cluster assembly in this bacterium [36]. This 25-amino-acid

residue stretch is absent in both SyCsd3 and AtNFS2 protein sequences (Figure 1B and the Results section). Furthermore, the three *Arabidopsis* IscU genes that can be retrieved from the genome sequence database (genes At4g04080, At4g22220 and At3g01020) encode putative proteins predicted to be targeted to mitochondria. It can be hypothesized therefore that if Fe–S cluster assembly occurs in plastids, it may involve an IscU-independent cyanobacterial-like machinery. Such a hypothesis is supported by the plastidial localization of an *Arabidopsis* IscA protein (accession number NP_172520), as determined by GFP fusion analysis (B. Touraine, S. Léon, J. F. Briat and S. Lobreux, unpublished work). This nuclear-encoded protein is highly related to *Synechocystis* PCC6803 IscA protein (accession number P72731) and to an IscA-like polypeptide encoded by the plastidial genome of the red alga *Porphyra purpurea* (accession number P51217). IscA proteins are involved in Fe–S cluster assembly [38–40], and have recently been proposed as alternative scaffolds to the IscU protein in this process [41].

Our data demonstrate that the recombinant AtNFS2 protein produced in *E. coli* bears a cysteine desulphurase activity (Figure 5), consistent with its sequence identity with other NifS-like proteins (Figure 1). Therefore, in order to check whether AtNFS2 cysteine desulphurase could be involved in Fe–S cluster assembly *in vivo*, we used the yeast *Anfs1* mutant (strain ×478-6c, kindly provided by Professor A. Dancis, Department of Medicine, Division of Hematology/Oncology, University of Pennsylvania, Philadelphia, PA, U.S.A.; [6]) in a functional complementation assay. This assay was based on the restoration of a wild-type phenotype to the yeast *Anfs1* mutant strain, through expression of a chimaeric construct encoding AtNFS2 with its plastid transit peptide sequence replaced by yeast ATP2p mitochondrial targeting sequence [42], so that the plant protein can be addressed to yeast mitochondria. However, no functional complementation was observed, even when the nuclear function of yeast NFS1p [43] – which cannot be complemented by AtNFS2 and which lacks a nuclear localization sequence – is restored by a prior transformation of *Anfs1* cells with a construct encoding a truncated nuclear form of yeast NFS1p (results not shown).

At this point, it cannot be ruled out that AtNFS2 function may be unrelated to Fe–S cluster biosynthesis. Only recently a NifS domain-containing enzyme has been shown to have a molybdenum cofactor-sulphurase activity in *A. thaliana* [44,45]. This protein, encoded by the *ABA3/LOS5* locus, shows similarities to NifS-like proteins in its N-terminal domain, and is involved in abscisic acid biosynthesis; the aldehyde oxidase involved in the last step of this pathway actually requires a sulphurylated molybdenum cofactor. This is consistent with the more general sulphur transferase activity of NifS-related enzymes. Actually, the NifS-like protein of *E. coli*, IscS, is also involved in the biosynthesis of sulphur-containing compounds such as the thiazole moiety of thiamin, or 4-thiouridine in thiolated tRNA [11]. Thiazole biosynthesis has been shown for a long time to occur in plastids, using cysteine as the sulphur donor [46]. More recent evidence indicates that many other plant biosynthetic enzymes involved in thiamin biosynthesis are either predicted or shown to be targeted to plastids [47–50]. Therefore AtNFS2 could play a role in thiamin metabolism through its sulphur transferase activity. Actually, the enzyme involved in sulphur transfer from cysteine to finally give thiazole is still unknown, and no data concerning thiazole biosynthesis in cyanobacteria is available. AtNFS2 could be a multifunctional enzyme, as is the case for IscS in *E. coli* [11].

A further characterization of AtNFS2 will be required to assess whether this plastidial cysteine desulphurase is involved in

Fe–S cluster assembly, and/or in thiamin metabolism. This would lead to a greater knowledge of sulphur metabolism in plastids.

Note added in proof (received 16 July 2002)

While this paper was in the press, we became aware that a sequence similar to that of AtNFS2 DNA had been deposited in the GenBank® Nucleotide Sequence Database under the accession number AF419347.

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