The Essential Cytosolic Iron-Sulfur Protein Nbp35 Acts without Cfd1 Partner in the Green Lineage*‡

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In photosynthetic eukaryotes assembly components of iron-sulfur (Fe-S) cofactors have been studied in plastids and mitochondria, but how cytosolic and nuclear Fe-S cluster proteins are assembled is not known. We have characterized a plant P loop NTPase with sequence similarity to Nbp35 of yeast and mammals, a protein of the cytosolic Cfd1-Nbp35 complex mediating Fe-S cluster assembly. Genome analysis revealed that NBP35 is conserved in the green lineage but that CFD1 is absent. Moreover, plant and algal NBP35 proteins lack the characteristic CXXC motif in the C terminus, though it is required for Fe-S cluster binding. Nevertheless, chemical reconstitution and spectroscopy showed that Arabidopsis (At) NBP35 bound a [4Fe-4S] cluster in the C terminus as well as a stable [4Fe-4S] cluster in the N terminus. Holo-AtNBP35 was able to transfer an Fe-S cluster to an apoprotein in vitro. When expressed in yeast, AtNBP35 bound 55Fe dependent on the cysteine desulfurase Nfs1 and was able to partially rescue the growth of a cfd1 mutant but not of an nbp35 mutant. The AtNBP35 gene is constitutively expressed in planta, and its disruption was associated with an arrest of embryo development. These results show that despite considerable divergence from the yeast Cfd1-Nbp35 Fe-S scaffold complex, AtNBP35 has retained similar Fe-S cluster binding and transfer properties and performs an essential function.

Proteins carrying iron-sulfur (Fe-S) clusters as cofactors are common in virtually all life forms. Fe-S proteins catalyze crucial steps in fundamental processes, including nitrogen fixation, respiration, photosynthesis, various metabolic pathways, and regulation of gene expression (1). The most common types of Fe-S clusters are the [2Fe-2S] and the cubane [4Fe-4S] form. Although Fe-S clusters can be assembled by chemical means, dedicated proteins for in vivo Fe-S protein maturation have been discovered over the past 10 years (2, 3). In plants assembly proteins have been localized to the plastids and mitochondria, the endosymbiotic organelles where photosynthesis and respiration take place, respectively (for review, see Refs. 4–6). The plastids contain all six proteins of the so-called SUF system plus NFU-type scaffolds. The mitochondrial matrix contains the ISC3 system (for iron-sulfur cluster assembly). Both systems appear to follow the same biochemical steps; (i) generation of persulfide by a cysteine desulfurase (the Nfs1-Isd11 complex in mitochondria; CpNifs in plastids), (ii) transfer of persulfide and combination with iron to form an Fe-S cluster on a scaffold protein (ISU proteins in mitochondria; NFU2 in plastids), (iii) transfer of the Fe-S cluster from the scaffold protein to a target protein (mediated by chaperones in the ISC system).

Important Fe-S enzymes are also found in the cytosol and nucleus (3, 4). In plants, these include DNA repair enzymes, RNA polymerase I and III (7), xanthine dehydrogenase, abscisic aldehyde oxidase (AAO3), and cytosolic aconitase. How these proteins obtain their Fe-S clusters is currently not known. By analogy with yeast, it is presumed that the mitochondria are required for cytosolic Fe-S cluster assembly, but supporting experimental evidence has not yet been provided. Bioinformatic analyses have revealed that sequence relatives of the yeast CIA proteins (for cytosolic iron-sulfur cluster assembly) are also present in the genomes of the model plant species Arabidopsis thaliana and the green alga Chlamydomonas reinhardtii (4, 8). In yeast and humans the CIA machinery includes two P-loop NTases, Cfd1 and Nbp35, which form a heterotetrameric complex and function as Fe-S scaffolds, mediating the de novo assembly of an Fe-S cluster and its transfer to target apoproteins (9–12).

Cfd1 and Nbp35 are classified as Mrp-like proteins that belong to the Mrp/NBP35 subfamily of P loop NTases (13). Although Cfd1 and Nbp35 share 49% sequence similarity, they can be distinguished as different types based on their N-terminal domains (Fig. 1B). A third type of Mrp-like protein, HCF101, was characterized in plastids and is involved in the assembly of Photosystem I, which has three [4Fe-4S] clusters (14, 15). Recently, we have identified a fourth type of eukaryotic Mrp-like protein associated with Fe-S protein assembly. This protein, named Ind1, is localized in the mitochondria and is required for the effective assembly of respiratory Complex I, which contains eight Fe-S clusters, in the yeast Yarrowia lipolytica (16).

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3 The abbreviations used are: ISC, iron-sulfur cluster (assembly); At, A. thaliana; CIA, cytosolic iron-sulfur cluster assembly; FL, full-length; GFP, green fluorescent protein; N, deletion of N-terminal domain; NBP, nucleotide binding protein; Sc, S. cerevisiae.
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To initiate studies on Fe-S protein assembly in the plant cytosol and analyze how this process may depend on the organelar assembly systems, we investigated two uncharacterized Mrp-like proteins in Arabidopsis. Here we show that NBP35 (gene locus At5g50960) is essential for embryo development and is localized in the cytosol, whereas the gene product of At4g19540, designated INDL for Ind1-like, was localized in the mitochondria. AtNBP35 bound [4Fe-4S] clusters that were readily transferred to an apoprotein in vitro, consistent with an Fe-S scaffold function.

EXPERIMENTAL PROCEDURES

Plant Material and Yeast Strains—The A. thaliana lines, SALK_054678 and SALK_056204, were obtained from the Nottingham Arabidopsis Stock Centre. The presence and position of the T-DNA insert in the NBP35 gene (At5g50960) was confirmed by PCR. Segregation analysis showed that the T-DNA was linked to the embryo lethality phenotype in the SALK_056204 line. Arabidopsis cell cultures were generated as described (17). Saccharomyces cerevisiae strain W303-1A (MATa, ura3-1, ade2-1, trpl1-1, his3-11,15, leu2-3112, his3-11,15, leu2-3112) served as wild type. The Gal-NFS1 strain and conditions for depletion of Nfs1 protein were described previously (18).

The following yeast plasmids were used: pRS416 (p416) containing the MET25 promoter for intermediate expression and pRS426 (p426) with the TDH3 promoter for high expression (19).

Cell Fractionation and Protein Blot Analysis—Arabidopsis cell cultures were used as source material to obtain highly purified mitochondria (20), nuclei (21), and a cytosolic fraction. For the latter, drained cells were squashed in one volume of cold nuclei isolation buffer (21) and centrifuged for 10 min at 13,000 × g, and the supernatant was collected. Chloroplasts were isolated from rosette leaves (22). Total protein extract from plant tissue was prepared by grinding the tissue with 1–2 volumes of cold 50 mM Tris-HCl, pH 8.0, 5% (v/v) glycerol, 1% (w/v) SDS, 10 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride followed by centrifugation at 13,000 × g for 15 min to remove cell debris. Protein concentrations were determined with Coomassie Dye Reagent (Bio–Rad). Thirty μg of protein was separated by SDS-PAGE, blotted, and labeled with antibodies following standard protocols.

Polyclonal antibodies were raised against purified His-AtNBP35 and His-AtINDL. Antibodies against AtISU1 were a kind gift from Stéphane Lobréaux (23), and antibodies against pea PETC were kindly provided by John C. Gray (24). The monoclonal antibody against Arabidopsis actin (MA1–744) was from Affinity BioReagents, Golden, CO; polyclonals against histone 3 (ab1791) were from Abcam, Cambridge, UK. Antisera against Nfs1 and Leu1 were described previously (18).

In Vitro Fe-S Assembly—The full-length (FL) AtNBP35 coding sequence or a version lacking the first 54 codons (∆N) was isolated from wild-type Arabidopsis cDNA and cloned into the pET15b expression vector (Novagen) in-frame with an N-terminal His tag. Protein overexpression and affinity purification were carried out following the manufacturer’s manuals. Chemical reconstitution of the Fe-S clusters was carried out in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI). In a typical experiment, 20–30 μM protein in 25 mM Tris-HCl, 150 mM NaCl, pH 8.0 (buffer A), was treated with 10 mM dithiothreitol (final concentration) at 15 °C for 1 h. The reconstitution was started by the addition of 10 mol eq of ferric ammonium citrate and Li2S followed by incubation at 15 °C for 3 h. To remove non-bound iron and sulfide, reconstituted AtNBP35 was passed through a Sephadex G25 gel filtration column using buffer A containing 1 mM dithiothreitol. The assembly of Fe-S clusters was ascertained by UV-visible spectroscopy (Jasco V-550). The quantification of iron and acid-labile sulfide and performance of in vitro cluster transfer assays were as previously described (11, 25).

Electron Paramagnetic Resonance (EPR) and Mössbauer Spectroscopy—Protein samples were reconstituted as described above. Low temperature X-band EPR spectra of samples reduced with 2 mM dithionite were recorded with a Bruker ESP 300E cw spectrometer equipped with a helium flow cryostat ESR910 (Oxford Instruments). For the preparation of Mössbauer samples reconstitution was carried out with 57FeSO4. The samples were concentrated with Vivaspin 30-kDa concentrator devices and frozen on a cold aluminum block in the anaerobic glove box. Mössbauer measurements and simulations were carried out as described in Barthelme et al. (26).

Miscellaneous Methods—Published methods used were transient expression of green fluorescent protein (GFP) fusion pro-
tein using vector pOL-GFP-S65C (27) and radiolabeling of yeast cells, protein extraction, and immunoprecipitation (18). Error bars represent the S.D. value.

RESULTS

NBP35 but Not CFD1 Is Conserved in the Green Plant Lineage—With the availability of recently sequenced genomes of plants and algae, we investigated the conservation of Mrp-like proteins and their domains in the green lineage. Three separate coding sequences for proteins with a Mrp family signature were usually found: one with a putative mitochondrial targeting sequence, designated INDL, the plastid HCF101, characterized by an N-terminal DUF59 domain, and an NBP35 homolog with an N-terminal Fe-S cluster domain (Fig. 1). No other sequence relative that could correspond to yeast Cfd1 was found in any of the six plant, one moss, and four green algal genomes analyzed. In addition, the predicted NBP35 proteins in the green lineage differed from those in the fungi/Metazoa group in the C-terminal region after the highly conserved VENMS motif (Fig. 1B and supplemental Fig. S1). Most notably, the C-terminal CXXC motif, which is critical for Fe-S cluster binding to Cfd1 and Nbp35 in yeast (4), is absent from plant and algal NBP35 proteins. Instead, a ∼35-amino acid insertion is found that is conserved in all Viridiplantae genomes investigated so far but that has no sequence similarity to other known protein motifs. As a consequence, only one C-terminal cysteine residue is conserved in all NBP35 proteins: Cys-234 in S. cerevisiae numbering, corresponding to Cys-220 in Arabidopsis NBP35. However, a number of semiconserved cysteine residues are found in the C-terminal 70-amino acid sequence of plant and algal NBP35 proteins. The absence of CFD1 genes in the green lineage suggests that the NBP35 protein may function as a homo-oligomer in the cytosol without a CFD1-like partner.

AtNBP35 Is a Soluble Cytosolic Protein—To determine the cellular localization of NBP35 (At5g50960) in the model plant Arabidopsis, the full coding sequence isolated from cDNA was fused in-frame to a C-terminal GFP sequence and transiently expressed using particle bombardment of Arabidopsis leaves. GFP fluorescence was found in the cytoplasm, which is appressed to the plasma membrane in the highly vacuolate, jigsaw-shaped epidermal cells (Fig. 2, A and B). Green fluorescence was also observed in the nucleus, which, however, may be due to diffusion, a recurrent problem in determining the localization of nuclear proteins as GFP fusions (28, 29). We, therefore, investigated the localization of endogenous AtNBP35 using cell fractionation and immunoblotting. Antibodies specific to AtNBP35 (see supplemental Fig. S2) recognized an ∼37-kDa protein in total plant extracts (Fig. 2C, inset), in agreement with the predicted molecular mass of 37,306 Da. The AtNBP35 protein was found in the soluble, cytosolic fraction but not in purified nuclei, mitochondria, or chloroplasts. We also investigated the localization of the INLD protein (At4g19540) using specific antibodies and obtained a signal in the purified mitochondrial fraction only, similar to the pattern observed for ISU1 (Fig. 2C). Note that these two proteins are not or are barely detectable in total extract due to their low abundance and the relatively minor contribution of mitochondria to total protein in plant extract. These data show that AtNBP35 is a soluble cytosolic protein that is spatially separated from the homologous AtINDL protein in the mitochondria.

Both the N- and C-terminal Domains of AtNBP35 Bind Fe-S Clusters—Because the characteristic C-terminal CXXC motif of the Cfd1/Nbp35 scaffold proteins is missing in plant and algal NBP35 proteins, it was of interest to analyze the capacity of the C-terminal domain to bind an Fe-S cluster. To this end, AtNBP35 was overproduced in Escherichia coli, either FL or lacking the N-terminal Fe-S cluster binding motif (ΔN). The proteins were purified by His-tag affinity purification (Fig. 3A, inset). The FL protein appeared brown in color, whereas the ΔN

4 D. J. A. Netz, unpublished information.
isolated by affinity chromatography. Spectra were recorded at protein concentrations of 28 μM (FL) and 47 μM (ΔN) and normalized to 25 μM for comparison. Dashed line, FL AtNBP35 reduced with 2 mM sodium dithionite. Inset, Coomassie staining of the purified protein samples separated by SDS-PAGE, showing only minor contaminating protein bands. B, UV-visible spectra of FL and ΔN AtNBP35 after reconstitution with sulfide and iron under anaerobic conditions. Spectra were recorded at protein concentrations of 20 μM (FL) and 31 μM (ΔN) and normalized to 25 μM for comparison. Inset, quantification of iron and acid-labile sulfide in reconstituted FL AtNBP35 (n = 3) and ΔN AtNBP35 (n = 2). C, comparison of the EPR signals of Arabidopsis NBP35, chemically reduced with 2 mM sodium dithionite, with the yeast and human Nbp35 proteins. EPR conditions: temperature, 10 K; microwave power, 2 milliwatts; microwave frequency, 9.459 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.25 millitesla (mT). D, Mössbauer spectrum recorded at 80 K (no applied field) of reconstituted FL AtNBP35. The lines are the simulated quadrupole doublets with parameters (δ, ΔE2; full width at half-maximum, all in mm/s): 0.43, 1.20, and 0.42 (85% of total intensity) and 0.34, 0.65, and 0.44 (15% of total intensity). The solid line is the sum of the simulated components. E, gel filtration of AtNBP35. His-tagged AtNBP35 (39.5 kDa) eluted in two peaks from a Superdex 200 column (GE Healthcare), corresponding to a dimeric form (calculated molecular mass of 98 ± 10 kDa) and a tetrameric form (188 ± 17 kDa).

FIGURE 3. AtNBP35 binds [4Fe-4S] clusters. A, UV-visible spectra of FL AtNBP35 and of ΔN AtNBP35 lacking the N-terminal Fe-S cluster binding motif (ΔN). The His-tagged proteins were overproduced in E. coli and isolated by affinity chromatography. Spectra were recorded at protein concentrations of 28 μM (FL) and 47 μM (ΔN) and normalized to 25 μM for comparison. Dashed line, FL AtNBP35 reduced with 2 mM sodium dithionite. Inset, Coomassie staining of the purified protein samples separated by SDS-PAGE, showing only minor contaminating protein bands. B, UV-visible spectra of FL and ΔN AtNBP35 after reconstitution with sulfide and iron under anaerobic conditions. Spectra were recorded at protein concentrations of 20 μM (FL) and 31 μM (ΔN) and normalized to 25 μM for comparison. Inset, quantification of iron and acid-labile sulfide in reconstituted FL AtNBP35 (n = 3) and ΔN AtNBP35 (n = 2). C, comparison of the EPR signals of Arabidopsis NBP35, chemically reduced with 2 mM sodium dithionite, with the yeast and human Nbp35 proteins. EPR conditions: temperature, 10 K; microwave power, 2 milliwatts; microwave frequency, 9.459 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.25 millitesla (mT). D, Mössbauer spectrum recorded at 80 K (no applied field) of reconstituted FL AtNBP35. The lines are the simulated quadrupole doublets with parameters (δ, ΔE2; full width at half-maximum, all in mm/s): 0.43, 1.20, and 0.42 (85% of total intensity) and 0.34, 0.65, and 0.44 (15% of total intensity). The solid line is the sum of the simulated components. E, gel filtration of AtNBP35. His-tagged AtNBP35 (39.5 kDa) eluted in two peaks from a Superdex 200 column (GE Healthcare), corresponding to a dimeric form (calculated molecular mass of 98 ± 10 kDa) and a tetrameric form (188 ± 17 kDa).

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B

C

D

E

protein was virtually colorless except for a light yellow hue. UV-visible spectroscopy of the FL protein showed absorbance peaks at 320 and 415 nm, which were more than halved upon addition of dithionite (Fig. 3A). These spectral features are characteristic of a [4Fe-4S] cluster (30); however, the intensity of the signal indicated that only a minor fraction of the molecules contained a cluster (11). The UV-visible spectrum of ΔN AtNBP35 (as isolated) did not have a pronounced absorbance. We, therefore, incubated both FL and ΔN protein with excess iron and sulfide under reductive, anaerobic conditions to chemically reconstitute the Fe-S clusters. Quantification of protein-bound Fe and sulfide showed that FL AtNBP35 bound 5.7 ± 1.2 iron and 5.1 ± 0.8 acid-labile sulfide per polypeptide, whereas the ΔN protein bound 1.8 ± 0.7 iron and 1.7 ± 0.1 acid-labile sulfide per polypeptide (Fig. 3B, inset). Subtraction of these numbers indicate that the N-terminal domain bound a [4Fe-4S] cluster. The C terminus may bind either a [2Fe-2S] cluster or a [4Fe-4S] cluster bridging two polypeptides, assuming complete reconstitution in our samples. The second possibility is supported by the UV-visible spectrum of reconstituted ΔN AtNBP35 (Fig. 3B), which is typical of a cubane cluster. Further support for a cubane cluster was obtained by EPR spectroscopy, showing attenuation of the EPR signal of the reduced protein above 40 K (supplemental Fig. S3).

The EPR spectrum of FL AtNBP35 after reduction with dithionite is rhombic and is dominated by the sharp signal of the N-terminal cluster, with g values of 2.051, 1.928, and 1.86 (Fig. 3C). In contrast, FL yeast Nbp35 and its human counterpart huNbp35 have an axial signal (Fig. 3C, see also Refs. 11 and 12). Comparison of the EPR signals of ΔN AtNBP35 (Fig. 3C) with the corresponding yeast ΔN Nbp35 and yeast Cfd1 (not shown) showed no pronounced differences for the relatively broad axial signal of the C-terminal domain. Mössbauer spectroscopy of the reconstituted protein (without reduction) showed that 85% of the iron associated with FL AtNBP35 was present in the form of [4Fe-4S]2+ clusters (Fig. 3D). The values of the isomer shift (0.43 mm/s) and quadrupole splitting (1.20 mm/s) were similar to the values observed for delocalized
mixed-valent (Fe$^{2.5+}$,Fe$^{2.5+}$) pairs in exclusively cysteine-coordinated [4Fe-4S]$^{2-}$ clusters (31). Because of the instability of ΔN AtNBP35 upon concentration of protein for Mössbauer spectroscopy, the individual isomer shift and quadrupole splitting parameters of N- and C-terminal clusters could not be determined.

Gel filtration analysis of recombinant as isolated FL AtNBP35 showed that most of the protein occurred as a dimer, with some forming a tetramer (Fig. 3E). The chromatographic behavior of ΔN AtNBP35 or the reconstituted proteins could not be analyzed due to instability during the gel filtration procedure. Taken together, these data indicate that AtNBP35 occurs as a homo-oligomer with a stable [4Fe-4S] cluster in the N-terminal domain of each subunit and a C-terminal [4Fe-4S] cluster shared between two protomers.

**Holo-AtNBP35 Can Transfer an Fe-S Cluster**—Next we investigated whether the Arabidopsis NBP35 protein can act as an Fe-S scaffold in that it can transfer an Fe-S cluster to a potential target protein in *vitro*. Following a previously described assay (11), chemically reconstituted and desalted FL AtNBP35 was added to apoLeu1, the apoform of yeast isopropylmalate isomerase. Cluster transfer was followed by the generation of enzymatic activity upon conversion of apoLeu1 to the [4Fe-4S]-containing form. Cluster transfer to Leu1 occurred at a rate and efficiency comparable with that observed for yeast Nbp35 or the Cfd1-Nbp35 complex (11). At the earliest time point that we were able to measure (1.5 min), more than 80% of the maximal Leu1 activity was obtained. In contrast, chemical reconstitution with the same amounts of ferric iron and sulfide hardly led to activation of apoLeu1 (Fig. 4). The addition of 1 mM ATP/GTP (plus MgCl$_2$) had no effect on cluster transfer, as has been noted for ApbC (40) and Cfd1/Nbp35.4 These *in vitro* data suggest that a labile Fe-S cluster of AtNBP35 can be donated to a target Fe-S protein.

**AtNBP35 Binds Fe-S Clusters in Vivo but Cannot Replace Yeast NBP35**—To analyze the physiological relevance of the physiological relevance of the in *vitro* data, we expressed AtNBP35 in yeast cells (*S. cerevisiae*, Sc). First, we confirmed that the AtNBP35 protein was localized in the yeast cytosol (Fig. 5A). Second, we investigated whether Fe-S clusters were assembled on AtNBP35 by the yeast Fe-S cluster assembly machinery. For this we made use of a mutant, Gal-NFS1, containing a galactose-regulatable NFS1 gene encoding the only cysteine desulfurase in yeast. Cluster assembly on AtNBP35 was followed by using a sensitive $^{55}$Fe radiolabeling assay (18). In brief, yeast cells overproducing AtNBP35 were incubated with $^{55}$Fe and washed followed by preparation of a soluble cell extract. AtNBP35 was immunoprecipitated from the cell extract and subjected to scintillation counting. A
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A significant amount of $^{55}$Fe (10 times over background) was associated with immunoprecipitated AtNBP35 (Fig. 5, B and C). Down-regulation of NFS1 expression led to a near loss of $^{55}$Fe bound to AtNBP35 (Fig. 5C, last bar). The Nfs1-dependent $^{55}$Fe binding to AtNBP35 indicates that the iron was part of an Fe-S cluster.

Next, we investigated whether AtNBP35 can replace the function of yeast Nbp35 using cells carrying a temperature-sensitive mutant copy of the endogenous NBP35 gene (32, 33). Upon shifting the cells to 37 °C, the mutant ScNbp35 protein becomes non-functional, leading to growth arrest after a few cell duplications. The growth of the temperature-shifted nbp35 mutant was rescued by expression of wild-type ScNbp35 from a plasmid but not by Arabidopsis NBP35 (Fig. 6). We then tested whether AtNBP35 could rescue a temperature-sensitive cfd1 mutant (33). Surprisingly, expression of AtNBP35 enabled slow growth of temperature-sensitive cfd1 mutant cells shifted to 37 °C. We also tested the functional requirement of the N-terminal domain of AtNBP35 for yeast complementation, as yeast Cfd1 lacks this domain. Truncated ΔN AtNBP35 did not complement the temperature-sensitive cfd1 mutant, although the protein was stably expressed (supplemental Fig. S2). Similar results were obtained with galactose-regulatable mutants of NBP35 and CFD1 (not shown).

Comparison of the signal intensities in lanes with cell extract and known amounts of purified protein indicated that the abundance of AtNBP35 is less than 0.005% of SDS-extractable protein.

Two plant lines with insertions in the AtNBP35 gene were obtained from the Arabidopsis stock center. SALK_054678 contains a T-DNA insert in the 5′-untranslated region, and SALK_056204 contains a T-DNA insert at the 3′ end of the first intron (Fig. 7B). Homozygous plants were bred from the SALK_054678 line, but they had no detectable phenotype. Reverse transcription PCR using primers in the 5′- and 3′-untranslated region confirmed the absence of full-length NBP35 transcript (not shown). However, PCR products covering the start and end of the coding sequence were as abun-
C-terminal C

cal reconstitution (Fig. 3). It will be interesting, therefore, to
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gal/metazoan NBP35 proteins is the absence of the C-terminal
dopsis (Fig. 2).

cytosolic isoform of the mitochondrial INDL protein in
sequence in any of the sequenced plant or algal genomes or as a
12) could not be identified either as a separate protein coding
AtNBP35, is present in the plant cytosol (Fig. 2). In contrast, a
Cfd1-type partner protein as found in fungi and Metazoa (11,
12) could not be identified either as a separate protein coding sequence in any of the sequenced plant or algal genomes or as a
cytosolic isoform of the mitochondrial INDL protein in Arabid-
opsis (Fig. 2).

Another marked difference between plant/algal and the fun-
gal/metazoan NBP35 proteins is the absence of the C-terminal
CXXC motif, which is replaced by an ~35-amino acid insertion
in the plant/algal proteins. This change may have occurred early in the evolution of the Viridiplantae, as it is found across
the green lineage (plants and green algae) but not in the rhodo-
phyte (red alga) Cyanidioschyzon merolae (39) (supplemental
Fig. S1). The C. merolae genome also encodes HCF101 and
INDL but not a CFD1 protein. Thus, the NBP35 protein in
C. merolae may represent the ancestral form containing the
C-terminal CXXC motif but not CFD1 partner. Hence, one
might speculate that CFD1 may have arisen as a consequence of
gene duplication in the fungal/metazoan group.

Despite the lack of the characteristic CXXC motif, the C-terminal domain of AtNBP35 bound an Fe-S cluster upon chemical
reconstitution (Fig. 3). It will be interesting, therefore, to
investigate more precisely the role of the CXXC motif in the
function of the Mrp-like proteins, as this motif is also absent in
HCF101. In contrast, mutation studies have shown that the
individual cysteines of the CXXC motif in yeast Cfd1 and Yap-
rowia Ind1 are essential (10, 16). Quantification of bound Fe
and sulfide in reconstituted AtNBP35 suggested that the C-ter-
inal [4Fe-4S] cluster was shared between two protomers. A
similar situation has been proposed for the bacterial Mrp-like
protein called ApbC in Salmonella enterica (40). As a conse-
quence, each polypeptide would provide two ligands to this
cluster which remain to be determined by site-directed
mutagenesis. The presence of the C-terminal cluster is not
required for dimer formation, as the as-isolated FL protein was
able to form dimers (Fig. 3, A and B).

To initiate the functional analysis of AtNBP35, we performed an in vitro cluster transfer assay and complementation studies
with yeast nbp35 and cfd1 mutants. Although the in vitro assay
suggested similar cluster transfer properties of AtNBP35 and
ScNbp35 (Fig. 4 and Ref. 11), expression of AtNBP35 could not
rescue the growth defect of ScNbp35-depleted cells (Fig. 6).
The reason for the lack of complementation is not known; Fe-S
cluster assembly on AtNBP35 could be demonstrated in yeast,
and control experiments confirmed stable expression of
AtNBP35 in the yeast cytosol (Fig. 5). Surprisingly, the growth of
ScCfd1-depleted cells was restored albeit partially by expres-
ion of FL AtNBP35 but not without the N-terminal domain.

Our studies presented here will be fundamental for further studies to unravel the precise molecular function of AtNBP35.
Because we have shown that AtNBP35 is an essential gene (Fig.
7), embryo lethality has to be circumvented by the construction of partial or regulatable knock-down mutants, which is in pro-
gress in our laboratory. Moreover, it will be interesting to inves-
tigate the cellular role of other CIA homologs in plants, includ-
ing AtNAR1 (41) and AtCIA1 (42), and to study their protein
interactions with AtNBP35. The presence of NBP35 as a homo-
oglomer in the plant cytosol rather than the Cfd1-Nbp35 com-
plex shows that plants provide an insightful model system for
Fe-S protein biogenesis and the compartmentalization of this
process in eukaryotic cells.

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spectrometer.

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Supplemental Figure S1 - Alignment of NBP35 amino acid sequences (ClustalW2)

Fe-S binding domain

<table>
<thead>
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<th>Species</th>
<th>Sequence</th>
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Mrp domain

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Conserved cysteine residues are highlighted in yellow.

Scer, *Saccharomyces cerevisiae* (Baker's yeast); Ylip, *Yarrowia lipolytica*; Hsap, *Homo sapiens* (human); Atha, *Arabidopsis thaliana* (thale cress); Vvin, *Vitis vinifera* (grape); Osat, *Oryza sativa* (rice); Zmay, *Zea mays* (mais); Cpar, *Cypripedium parviflorum* (orchid); Ptri, *Populus trichocarpa* (poplar tree); Psit, *Picea sitchensis* (sitca spruce); Ppat, *Physcomitrella patens* (moss); Ostr, *Ostreococcus lucimarinus* (green alga); Cmer, *Cyanidioschyzon merolae* (red alga).

The NBP35 sequences in the algae *Chlamydomonas reinhardtii* and *Volvox carteri* are available but have been omitted from this alignment for the following reason. Although it is clear that both sequences lack the C-terminal CxxC motif and have an insertion that shows similarity to the plant, moss and algae "APEL" domain, the length of the insertion is uncertain. From the only EST sequence that covers this region in Chlamydomonas, and our own preliminary RT-PCR data, it is likely that the "APEL" insertion is much longer (~70 amino acids) than in other viridiplantae NBP35s.
Supplemental Figure S2 – Stable expression of AtNBP35 in yeast and its detection by specific antibodies

AtNBP35 was cloned into the p416 plasmid, either the full-length (FL) coding sequence or lacking the first 54 codons (ΔN). The plasmids, as well as an empty p416 control, were transformed in yeast. Extracts of the transformed yeast cell lines were prepared and subjected to immunoblot analysis with antibodies raised against recombinant, purified His-tagged AtNBP35 protein. The antibodies specifically detected proteins of expected molecular mass (39.5 kDa including the His-tag for FL AtNBP35, 34.0 kDa for ΔN AtNBP35), but not the endogenous yeast Nbp35 protein (35.3 kDa).
EPR spectra of full-length AtNBP35 and ΔN AtNBP35 (chemically reconstituted) at 10, 25 and 40 Kelvin.
Gene expression of Arabidopsis *NBP35* (At5g50960) in various plant tissues. The data were retrieved from GENEVESTIGATOR, a database of micro-array data (Zimmermann et al., 2004. *Plant Physiol.* **136**, 2621-2632). The values shown are means + SD.