NBP35 interacts with DRE2 in the maturation of cytosolic iron-sulphur proteins in Arabidopsis thaliana

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SUMMARY
Proteins of the cytosolic pathway for iron-sulphur (FeS) cluster assembly are conserved, except that plants lack a gene for CFD1 (Cytosolic FeS cluster Deficient 1). This poses the question of how NBP35 (Nucleotide-Binding Protein 35 kDa), the heteromeric partner of CFD1 in metazoa, functions on its own in plants. Firstly, we created viable mutant alleles of NBP35 in Arabidopsis to overcome embryo lethality of previously reported knockout mutations. RNAi knockdown lines with less than 30% NBP35 protein surprisingly showed no developmental or biochemical differences to wild-type. Substitution of Cys14 to Ala, which destabilized the N-terminal Fe₄S₄ cluster in vitro, caused mild growth defects and a significant decrease in the activity of cytosolic FeS enzymes such as aconitase and aldehyde oxidases. The DNA glycosylase ROS1 was only partially decreased in activity and xanthine dehydrogenase not at all. Plants with strongly depleted NBP35 protein in combination with Cys14 to Ala substitution had distorted leaf development and decreased FeS enzyme activities. To find protein interaction partners of NBP35, a yeast-two-hybrid screen was carried out that identified NBP35 and DRE2 (Derepressed for Ribosomal protein S14 Expression). NBP35 is known to form a dimer, and DRE2 acts upstream in the cytosolic FeS protein assembly pathway. The NBP35–DRE2 interaction was not disrupted by Cys14 to Ala substitution. Our results show that NBP35 has a function in the maturation of FeS proteins that is conserved in plants, and is closely allied to the function of DRE2.

Keywords: Fe–S cofactor, cytosol, leaf development, aconitase, aldehyde oxidase, DNA methylation, CIAPIN1, yeast-two-hybrid, Arabidopsis thaliana.

INTRODUCTION
Iron–sulphur (FeS) clusters are versatile metal cofactors involved in electron transfer, catalytic reactions as well as regulation of gene expression (Beinert, 2000; Lill, 2009). The rhombic Fe₂S₂ and the cubane Fe₄S₄ clusters are the most common forms, liganded to the protein by three to four cysteine residues. In some FeS proteins, histidine or aspartate are used as ligands in addition to cysteines. FeS clusters are synthesized by dedicated assembly proteins in the plastids, mitochondria and cytosol. Plastids contain the SUF pathway, and mitochondria contain the ISC pathway, each involving at least eight proteins (Couturier et al., 2013; Balk and Schaedler, 2014). The assembly process starts with the formation of a persulphide on cysteine desulphurase, which is transferred to a scaffold protein. The persulphide (S₅) is reduced to sulphide (S²⁻) and combined with iron before transfer of the cluster to a target protein. The cytosol and nucleus harbour another set of seven to eight proteins required for FeS cluster assembly, known collectively as the CIA pathway for Cytosolic Iron–sulphur protein Assembly. The assembly process is dependent on the mitochondria, which are thought to provide persulphide, exported in the form of glutathione trisulphide by the ABC transporter Atm1 in yeast (Saccharomyces cerevisiae) or ATM3 in plants (Schaedler et al., 2014). It is still unknown how the iron is provided and incorporated in FeS clusters.

The proteins of the CIA pathway have been studied mostly in yeast (Netz et al., 2014), but are found in virtually all eukaryotes (Tsaousis et al., 2014). The first identified were two P loop NTPases, Cfd1 and Nbp35, that form a heterotetrameric complex (Netz et al., 2007). However, plants and algae lack a CFD1 gene, and purified Nucleotide-Binding Protein 35 kDa (NBP35) forms a homodimer
(Bych et al., 2008; Kohbushi et al., 2009). Arabidopsis NBP35 could not replace the yeast homologue in complementation assays, but in vitro data suggest that Arabidopsis NBP35 has a scaffold function similar to the yeast Npb35/Cfd1 complex. Specifically, the C-terminus of NBP35 binds a labile FeS cluster that can be transferred to an apoprotein (Netz et al., 2007; Bych et al., 2008). A second, stable ferredoxin-type FeS cluster is bound to the N-terminal domain of NBP35.

The other CIA proteins can be divided into two functional units based on protein interactions. A complex of four proteins, Nr1, Cia1, Cia2 and Met18 (yeast nomenclature), has been placed experimentally downstream of Nbp35/Cfd1. Cia2 and Met18 interact directly with FeS apoproteins in yeast, mammals and plants (Netz et al., 2014; Duan et al., 2015; Wang et al., 2016). Functioning upstream of Nbp35/Cfd1 is a complex of two CIA proteins, the diflavin reductase Tah18 and the FeS-binding Dre2 (Derepressed for Ribosomal protein S14 Expression, also known as Anamor- sin or CIAPIN1). They have a redox function with electrons passing from NADPH via Tah18 to Dre2.

There are only a limited number of functional studies on CIA proteins in plants because all genes except MET18 are essential. A non-lethal mutation in the CIA2/AE7 gene was found in a screen for mutants with altered leaf polarity (Yuan et al., 2010). ae7 mutants also have decreased activities of cytosolic and nuclear FeS enzymes, and increased DNA damage (Luo et al., 2012). met18 mutants do not have a visible growth phenotype, but are deficient in demethylating DNA as a result of decreased activity of the DNA glycosylase ROS1, a Fe₄S₄ enzyme, and possibly other demethylation pathways (Duan et al., 2015; Wang et al., 2016).

Here we present a viable mutant allele of NBP35 in Arabidopsis, and show that the protein is required for cytosolic aconitase (ACO) and aldehyde oxidase (AldOx) activities, but that DNA glycosylase and xanthine dehydrogenase (XDH) are less affected. The function of NBP35 is necessary during reproductive and vegetative development, and affects leaf polarity. In a yeast-two-hybrid screen with an Arabidopsis cDNA library, DRE2 was revealed as a protein interaction partner of NBP35.

RESULTS

Less than 30% NBP35 protein is sufficient for vegetative growth and the maturation of cytosolic FeS proteins

Previous studies showed that T-DNA insertion in the coding sequence of the NBP35 gene is embryonic lethal, for instance in the nbp35-1 and nbp35-2 mutant alleles (Figure 1a; Bych et al., 2008; Kohbushi et al., 2009). In contrast, the nbp35-3 allele, which has a T-DNA insertion in the 5’UTR (-49 nt), is viable (Bych et al., 2008; Nakamura et al., 2013). Using specific antibodies, we found that NBP35 protein levels in leaves of nbp35-3 are 47 ± 13% of wild-type levels, but the plants have no obvious phenotype under standard growth conditions (Figure 1b and c).

To obtain alleles with a stronger depletion of NBP35, we silenced expression using an ethanol-inducible RNAi sequence. Of 12 independent lines, two were selected for...
further study because of a strong decrease in NBP35. Leaf extracts of RNAi plants treated with ethanol for 2 weeks contained 29 ± 5% NBP35 of wild-type levels as measured by semi-quantitative protein blot analysis with NBP35-specific antibodies (Figures 1c and S1). NBP35-depleted plants looked similar to wild-type or water-treated controls (Figure 1b). To investigate if there was any effect on the maturation of cytosolic FeS enzymes, we analysed the levels of ACO by immunolabelling and the activities of AldOx by in-gel staining (Figure 1c). Neither ACO nor AldOx were decreased in the RNAi lines or in nbp35-3 compared with wild-type leaves.

Cys14 to Ala substitution in NBP35 decreased the activities of selected FeS enzymes in the cytosol

As an alternative approach to create a viable mutant allele of NBP35, we decided to replace the endogenous NBP35 protein with a mutant version. We targeted amino acid residues that are evolutionary conserved but not essential in yeast Nbp35 (Netz et al., 2012). Cys27 is a ligand of the N-terminal FeS cluster in yeast and aligns with Cys14 in Arabidopsis NBP35 (Figures 2a and S2), which was changed to alanine using site-directed mutagenesis (C14A). Heterozygous nbp35-1/+ plants were transformed with NBP35-C14A and with wild-type NBP35 as a control. We used three different promoters to drive expression of the transgene: the CaMV 35S promoter; the constitutive UBQ11 promoter; and the endogenous NBP35 promoter. T1 seeds were germinated on selective medium, and plants were genotyped by polymerase chain reaction (PCR). We were unable to find any homozygous nbp35-1 plants with 35S:NBP35 or 35S:NBP35-C14A in the T1 or T2 generation (Table 1). The activity of the CaMV 35S promoter in Arabidopsis is low in

Figure 2. Cys14 to Ala substitution in NBP35 leads to decreased FeS enzyme activities.
(a) Schematic of NBP35 protein domain structure including the N-terminal and C-terminal cysteine motifs flanking the P-loop domain. (b) Leaf rosette development in 25-day-old plants with either NBP35 or NBP35-C14A expressed from the UBQ11 promoter in the absence of endogenous NBP35 (homozygous for nbp35-1). Plants were grown under long-day conditions. Scale bar: 1 cm. (c) Protein blot analysis of NBP35 and aconitase (ACO), as well as activity staining for ACO, aldehyde oxidase (AldOx) and xanthine dehydrogenase (XDH) activities in wild-type (WT) and three independent lines each of UBQ11:NBP35 and UBQ11:NBP35-C14A in the nbp35-1 background. (d) McrBC-PCR assay on genomic DNA samples from the indicated lines using two selected loci (MRD1 and XBAT34). Lack of a PCR product after digestion with the methylation-dependent endonuclease McrBC (+) indicates hyper-methylation due to inactive ROS1. The ros1-3 mutant was included for comparison. PCR with ACTIN8-specific primers served to confirm the levels of genomic DNA.
female organs and absent from pollen (Wilkinson et al., 1997); therefore, the inability of 3SS:NBP35 to complement the npb35-1 knockout allele suggests an essential function of NBP35 in reproductive tissues.

Expression of NBP35 or NBP35-C14A driven by the UBO11 promoter rescued seedling lethality of the npb35-1 mutation (Table 1). The UBO11:NBP35 complemented plants were indistinguishable from wild-type. In contrast, the NBP35-C14A lines displayed subtle phenotypes such as enlarged leaves (Figure 2b) and ~50% shorter roots in one of the lines (Figure S3a). Protein levels of both NBP35 and NBP35-C14A were increased ~twofold compared with wild-type leaves, indicating that the amino acid change does not affect the stability of NBP35 (Figure 2c).

Next, we analysed a range of FeS enzymes for protein stability and/or activity. ACO protein levels were decreased in all three independent UBQ11:NBP35-C14A lines but not in the UBO11:NBP35 lines, suggesting that impaired function of NBP35 causes protein instability of ACO due to lack of the Fe₄S₄ cofactor. To separate out mitochondrial and cytosolic isoforms of ACO, cell extracts were analysed using an in-gel activity assay (Bernard et al., 2009). This showed that cytosolic but not mitochondrial ACO activity was decreased in the UBO11:NBP35-C14A lines (Figure 2c). The transcript levels of ACO1, encoding the cytosolic isoform of ACO in the leaves, were generally increased in the C14A lines compared with wild-type (Figure S3b). The activities of AldOx and XDH, which bind two Fe₄S₄ clusters, were also analysed by in-gel assays. AldOx activities were decreased as a result of NBP35-C14A, but XDH activity was not (Figure 2c). The Fe₄S₄-dependent protein ROS1 is part of a small gene family of DNA glycosylases that removes methylated cytosines. The activity of ROS1 can be determined using a PCR assay in combination with a methylation-dependent restriction enzyme on genomic DNA sequences that are known to be demethylated by ROS1. In all three UBO11:NBP35-C14A lines the ROS1 target XBAT34 was hypermethylated, but another target MRD1 was not affected (Figure 2d).

To ensure that FeS enzyme defects were not a result of increased oxidative stress or altered metal homeostasis, the activities of superoxide dismutases (Fe, Mn and CuZn isoforms) and catalase (haem) were assessed. Neither superoxide dismutase nor catalase activities were affected by mutation of NBP35. The activities of superoxide dismutases (Fe, Mn and CuZn isoforms) and catalase (haem) were assessed. Neither superoxide dismutase nor catalase activities were affected by mutation of NBP35. To ensure that FeS enzyme defects were not a result of increased oxidative stress or altered metal homeostasis, the activities of superoxide dismutases (Fe, Mn and CuZn isoforms) and catalase (haem) were assessed. Neither superoxide dismutase nor catalase activities were affected by mutation of NBP35.
homozygous for npb35-1 (Table 1). Extensive genotyping of offspring from npb35-1/+ plants carrying the NBP35-C14A transgene identified a low frequency of npb35-1/+ segregants. Growth was extremely delayed (Figure 4a) and leaf development was abnormal, with irregular arrangement of the rosette leaves and asymmetric or even forked leaf blades (Figures 4b and S4). Further analysis of leaf anatomy showed no midvein and a loss of adaxial-abaxial polarity. The palisade parenchyma on the adaxial side was not formed in NBP35:NBP35-C14A plants and large intercellular spaces were found throughout. Moreover, cell size was increased (Figure 4d), similar to atm3-1 (starik; Kushnir et al., 2001) and ae7 mutants (Yuan et al., 2010). Plants bolted after ~12 weeks from leaf axils. The racemes were highly branched and compact carrying short siliques with approximately four seeds (Figures 4c and S4). Interestingly, most of the phenotypes described above were not observed in the next generation (Figure 4a), only the larger leaves as seen in the UBQ11:NBP35-C14A line (Figure 2b).

Protein blot analysis showed that NBP35-C14A levels in the npb35-1/+ segregants were strongly decreased compared with the hemizygous npb35-1/+ (Figure 4e). This may be due to regulatory sequences in the promoter or 3′UTR that were not included in the transgene. Indeed, NBP35:NBP35 lines also had lower levels of NBP35 protein than wild-type (Figure S4f). In NBP35:NBP35-C14A plants, the FeS enzymes ACO and AldOx were much reduced in levels or activities (Figure 4e). These data suggest that NBP35 fundamentally contributes to establishing cell size and leaf polarity, most likely because FeS proteins are required for these processes.

C14A substitution destabilized the N-terminal Fe₄S₄ cluster of NBP35

To investigate the effect of C14A substitution on FeS cluster binding, NBP35 and NBP35-C14A were overproduced in Escherichia coli and purified, aerobically, by His-tag affinity purification (Figure S5). The freshly isolated wild-type protein had a pale reddish-brown colour, whereas the C14A form was nearly colourless. UV-visible spectroscopy of wild-type NBP35 displayed a broad shoulder at 410 nm (Figure 5a) as previously observed, consistent with the presence of FeS clusters (Bych et al., 2008; Kohbushi et al., 2009). For the C14A protein, the spectrum was similar to wild-type NBP35 but the absorbance at 410 nm was sixfold less, indicating changes in cluster assembly and/or stability. We noted that the ‘as isolated’ wild-type NBP35 spectrum is reminiscent of a mixture of Fe₂S₂ and Fe₄S₄ clusters, most likely a result of cluster degradation (Tucker et al., 2008; Kohbushi et al., 2009; Crack et al., 2015).

Following in vitro anaerobic cluster reconstitution, samples of wild-type and C14A NBP35 yielded darker, brown-coloured solutions with UV-visible absorbance spectra containing a broad shoulder at 400–420 nm together with a less well resolved feature at ~310 nm, indicative of the presence of Fe₄S₄ clusters (Figure 5b). The magnitude of the absorbance at 410 nm indicated approximately 30% of cluster occupancy. Because FeS clusters derive their optical activity from the fold of the protein to which they are ligated, circular dichroism (CD) spectroscopy can provide information about the cluster environment. The anaerobic CD spectrum of reconstituted wild-type NBP35 displayed positive (+) features at 328, 374, 400 and 482 nm, together with negative (−) features at 444 and 550 nm. The CD spectrum of NBP35-C14A displayed positive (+) features at 319 and 434 nm, and negative (−) features at 378 and 537 nm (see Figure S6b). Overall, the CD spectra are distinct, indicative of significant differences in the local protein environment and ligation pattern of the Fe₄S₄ clusters in the two proteins.

To assess the stability of the Fe₄S₄ clusters, wild-type and C14A NBP35 were exposed to dissolved atmospheric oxygen, and the absorbance at 406 nm (A₄06) was followed over time. Wild-type NBP35 displayed a 10% loss in A₄06 over 20 min (Figure 5c), with little change in its absorbance and CD spectra (Figure S6). In contrast, NBP35-C14A displayed a 30% loss in A₄06 over the same time period, together with changes in the absorbance and CD spectra that are consistent with a mixture of Fe₂S₂ and Fe₄S₄ clusters (Figures 5c and S6). Taken together, the data demonstrate that C14A substitution destabilizes the binding of the N-terminal Fe₄S₄ cluster of Arabidopsis NBP35.

Arabidopsis NBP35 physically interacts with DRE2

NBP35 is likely to interact with upstream and downstream proteins of the CIA pathway, but such interactions have not been identified (Paul et al., 2015). To identify interaction partners of NBP35 in Arabidopsis, a yeast-two-hybrid screen was carried out using N-terminally fused NBP35 as bait (BD-NBP35) and an Arabidopsis seedling library of prey fragments. Of 47.8 million fragments screened there were 331 positive hits for 54 different proteins (Table S1). The results were filtered for proteins with more than one hit that were predicted or confirmed to have a cytosolic localization (http://suba3.plantenergy.uwa.edu.au/). Among the resulting eight proteins (Table 2) was NBP35, which was expected because NBP35 has been shown to form a dimer (Bych et al., 2008; Kohbushi et al., 2009) and therefore served as a positive control. For the remaining seven proteins, we considered expression patterns during Arabidopsis development (Winter et al., 2007). This showed that only the expression pattern of DRE2 (AT5G18400) correlated with that of NBP35 (Figure S7).

To confirm the protein interaction between NBP35 and DRE2, full-length NBP35 was used as bait and full-length DRE2 was used as prey with a different yeast-two-hybrid system (INVITROGEN ProQuest kit). DRE2 could not be used as bait because this auto-activated the reporter genes. Yeast growth on selective medium demonstrated...
The strength of the NBP35–DRE2 protein interaction was compared with the previously reported DRE2–TAH18 physical interaction (Varadarajan et al., 2010) using 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the protein product of the HIS3 gene. Growth of yeast depending on the DRE2–TAH18 protein interaction was much more robust than for the NBP35–DRE2 combination, suggesting that the binding affinity of DRE2 for NBP35 is relatively weak compared with TAH18 (Figure 6a).

The interaction between NBP35 and DRE2 was further tested using Surface Plasmon Resonance (SPR). Strep-tagged DRE2 was immobilized on a Streptavidin chip and the binding of increasing concentrations of purified NBP35 (0–45 μM) was recorded in a multi-cycle experiment. The positive response during injection of NBP35 (Figure 6b, first 60 s) showed that NBP35 bound to DRE2. The dissociation of NBP35 was slow, suggesting a relatively strong binding once NBP35 and DRE2 interact. Fitting the response to the concentrations of NBP35 gave an affinity of \(3.3 \pm 0.9\) μM (Figure 6c). Taken together, the results from yeast-two-hybrid and SPR experiments show a specific protein interaction between NBP35 and DRE2.

We also investigated protein interaction between NBP35 and NAR1 from Arabidopsis, a protein downstream in the CIA pathway. Yeast-two-hybrid analysis with NBP35 as prey and NAR1 as bait did not show an interaction (Figure 6a). It is possible that the observed interactions between Arabidopsis CIA proteins could be affected by the presence of yeast homologues, but we think this is unlikely because Arabidopsis NBP35 cannot complement yeast nbp35 mutants (Bych et al., 2008; Kohbushi et al., 2009) and Arabidopsis DRE2 cannot complement a yeast dre2 mutant unless co-expressed with Arabidopsis TAH18 (Bernard et al., 2013).

Because C14A substitution in NBP35 decreases the activity of FeS enzymes, we tested the effect of this substitution in the yeast-two-hybrid assay. We found that interaction between NBP35-C14A and DRE2 was similar to the interaction between wild-type NBP35 and DRE2 (Figure 6d). This indicates that cluster binding to the N-terminal domain is not required for the interaction.

**DISCUSSION**

Proteins of the CIA pathway are highly conserved in eukaryotes (Figure S9), but they remain relatively
understudied. Both the essential nature of most CIA genes and low levels of expression are likely to contribute to their elusiveness. In plants, several CIA genes have recently been identified in mutant screens for gene silencing by DNA methylation: AE7/CIA2, DRE2, NAR1 and MET18 (Yuan et al., 2010; Nakamura et al., 2013; Buzas et al., 2014; Duan et al., 2015; Wang et al., 2016). Because a comprehensive phenotypic analysis has only been carried out for AE7 (Luo et al., 2012), we undertook a study to define the role of Arabidopsis NBP35 in the maturation of FeS enzymes and in plant development.

Depletion of NBP35 to less than 30% of wild-type protein levels did not result in any obvious phenotype or in decreased FeS enzyme activities in the leaves (Figures 1b and c, and S1). Either NBP35 protein levels are in excess in the vegetative stage, or the effect on FeS enzymes is indirect, via another process important for FeS cluster assembly. The homologous INDH protein in mitochondria is also thought to play an indirect role in the assembly of the eight FeS clusters of complex I, although its precise function has not been identified yet (Wydro et al., 2013). In contrast to the RNAi lines, stable expression of a mutated version of NBP35 showed a clear decrease in a number of cytosolic FeS enzymes, namely ACO, AldOx and, to some extent, the DNA glycosylase ROS1 (Figure 2c and d). The relatively mild nature of the C14A substitution may explain

<table>
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<th>Cellular localization</th>
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47.8 million clones from an Arabidopsis seedling cDNA library were screened, resulting in 331 hits for 54 different gene fragments. Those coding sequences with ≥ 2 hits for proteins with a cytosolic localization are listed here. The full list can be found in Table S1.

*Based on http://suba3.plantenergy.uwa.edu.au/.

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that not all FeS enzymes are equally affected, with XDH not affected at all. At the same time, the results indicate a certain priority in the maturation of FeS proteins, also observed in ae7 Arabidopsis mutants and in human cells depleted of the NBP35 homologue (Stehling et al., 2008).

Low levels of NBP35-C14A resulted in severe developmental defects, which were evidently suppressed by over-expression of NBP35-C14A using the UBQ11 promoter (Figures 3a–c and S4). The asymmetric leaves lacked the differentiated cell layers that define the upper (adaxial) and lower (abaxial) side of the leaf, the palisade and spongy parenchyma, respectively. Moreover, cells were enlarged in the mutants. The change in anatomy and cell size indicates a problem with cell division and establishment of tissue polarity. Interestingly, this has also been observed in nbp35 (nubp1) mutants in mouse during lung development and ciliogenesis (Schnatwinkel and Niswander, 2012; Kypri et al., 2014; Ververis et al., 2016). Identification of a protein interaction between Nubp1 and the kinesin-like protein (KIFC5A) involved in bipolar spindle assembly further implicated Nubp1 in the cell cycle (Christodoulou et al., 2006). Alternatively, a defect in ribosome function could also lead to aberrant leaf development as shown for a mutant in RNase L inhibitor 2 (RLI2) in Cardamine hirsuta, a plant species related to Arabidopsis (Kougioumoutzi et al., 2013). RLI is highly conserved in eukaryotes and Archaea, including its two Fe_{2}S_{2} cluster binding motifs, and is essential for ribosome assembly. It is interesting to note that many of the CIA proteins have been identified in screens for ribosome mutants, including the DRE2 gene in yeast.

While the first generation of NBP35:NBP35-C14A plants had severe developmental phenotypes, the few seeds they produced developed normally except that the plants were significantly bigger in size. A possible explanation of this unusual observation is that the limited function of NBP35 causes genome instability, as found in ae7 mutants (Luo et al., 2012), which could disrupt meiosis and prevent fertilization. The few seeds that survive may have one or more suppressor mutations, enabling vigorous growth in the next generation. Whole genome sequencing of first- and second-generation plants could be used to identify such mutations.

In yeast, mammals and other metazoa NBP35 has been shown to interact consistently with CFD1/Nubp2. No interactions between NBP35 and other CIA proteins have been found so far, suggesting that such interactions may be transient, reliant on FeS occupancy and/or require binding of multiple proteins. The absence of CFD1 in plants may be the reason why we were able to detect DRE2 as an interaction partner of NBP35 (Figures 6 and S8; Table S1). DRE2 has already been shown to interact with the flavoprotein TAH18 (Varadarajan et al., 2010), forming an electron transport chain in an early step required for FeS protein assembly.
Molecular cloning, site-directed mutagenesis and plant transformation

For downregulation of NBP35 expression by RNAi, 121 nucleotides of the NBP35 coding sequence (ATG5G0980, c.517–627) were placed in sense and antisense orientation under the control of the ethanol-inducible alcR-alcA gene expression system (Roslan et al., 2001). Homozygous plants from the T3 generation were sprayed with 2% (v/v) ethanol from day 14, every other day, and tissue was harvested between 4 and 5 weeks.

For amino acid substitution, the NBP35 coding sequence was PCR-amplified from cDNA and cloned behind the CaMV 35S promoter using NcoI and XbaI in a pUC-derived cloning plasmid. Similarly, the coding sequences of NBP35-C14A constructs. Heterozygous nbp35-1/− plants were transformed by the floral-dip method using the Agrobacterium tumefaciens strain GV3101. Successfully transformed plants were selected on ½ MS with 0.8% (w/v) agar plus 25 µg mL−1 hygromycin. Plants were genotyped for the presence of the NBP35 transgene using exon primers EB21 and EB22 that span an intron of NBP35. Zygosity of the nbp35-1 allele was determined using the primers EB77 (LBb1.3) in the T-DNA and EB32 in the NBP35 gene.

**Protein blot analysis**

Protein extracts were separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred under semi-dry conditions to nitrocellulose membrane for immunolabelling. Ponceau-S staining of the membranes was used to confirm equal protein loading and successful transfer. Polyclonal antibodies against Arabidopsis NBP35 and ACO were as previously described (Bych et al., 2008; Bernard et al., 2009).

**Enzyme assays**

In-gel assays for AldOx, XDH and ACO were as previously reported (Bernard et al., 2009). Catalase activity was measured using a spectroscopic assay for H2O2 (Beers and Sizer, 1952). Superoxide dismutase activity was measured according to Chu et al. (2005). The McrBC-PCR assay was performed as previously described (Luo et al., 2012) using specific primers for the XBAT34 and MDR1 loci (Table S2).

**Microscopy**

Leaf material was fixed with a solution of 3.7% (w/v) formaldehyde, 5% (v/v) acetic acid, 50% (v/v) ethanol and embedded in LR white. Transverse sections (0.5 µm) were stained with toluidine blue and viewed on a LEICA DM6000 light microscope. The objectives used were ×100/0.4 air, ×20/0.7 air or ×40/0.95 air, and overview images were collected by taking a series of partially overlapping images with a LEICA DFC420c colour camera, which were merged using LEICA LAS-ADF software.

**Protein interaction assays**

The ULYTmate Y2H screen was carried out by HYBRIDGENICS SERVICES (Paris, France) using an A. thaliana seedling cDNA library (ecotype Columbia, 1-week-old seedlings). NBP35 was used as bait and N-terminally fused to the DNA-binding domain. To test binary interactions the INVITROGEN ProQuest Two-Hybrid System with Gateway Technology was used. The coding sequences of Arabidopsis NBP35, DRE2, TAH18 and NAR1 were N-terminally fused to the DNA-binding domain (BD) and/or DNA-activation domain (AD) as indicated. The BD-DRE2 protein auto-activated transcription and was therefore not used.
Surface Plasmon Resonance was performed using a GE HEALTHCARE (Little Chalfont, UK) Biacore T200 instrument with a Streptavidin sensor chip. After washing with 1 M NaCl, 50 mM NaOH at a flow rate of 10 μL min⁻¹ to remove any unconjugated Streptavidin, E. coli cell extract containing Strep-tagged DRE2 (Bernard et al., 2013) was allowed to flow over the surface at a flow rate of 30 μL min⁻¹ for 30 sec. A response of approximately 1700 response units was obtained, indicating that DRE2 was bound to the chip. Purified NBP35 was diluted in running buffer (20 mM Tris- HCl pH 8, 0.15 M NaCl, 5% glycerol) and injected over immobilized DRE2-Strep and over a control flow cell without DRE2-Strep. Multi-cycle binding experiments were performed at 25°C at a flow rate of 30 μL min⁻¹. Each concentration of NBP35 was injected for 60 sec and then switched to buffer flow to monitor the dissociation for 60 sec. The chip surface was regen-erated by one 30-sec injection of 1 M NaCl, 10 mM NaOH followed by washing with running buffer for 60 sec. Non-selective binding to the chip surface was accounted for by subtracting the response in the control flow cell. The average equilibrium response taken 4 sec before the end of the injection was plotted against NBP35 concentrations using the Affinity Fit option from the Biacore T200 evaluation software v2.0 assuming a 1:1 binding model.

Reconstitution of FeS clusters, and UV-visible absorbance and CD spectroscopies

The full-length Arabidopsis NBP35 coding sequence was cloned into the pET15b expression vector (NOVAGEN) in-frame with an N-terminal His tag (Bych et al., 2008). Site-directed mutagenesis was used to introduce a Cys to Ala substitution at position 14. Protein overexpression and affinity purification were carried out following the manufacturer’s instructions using a His-Trap HP column (GE HEALTHCARE) on an AKTA Pure system with a linear gradient of imidazole. NBP35 (wild-type or C14A)-containing fractions were immediately desalted with 20 mM Tris-HCl pH 8, 150 mM NaCl and 5% (v/v) glycerol (wash buffer) using a PD10 column (GE HEALTHCARE) and a sixfold molar excess of ferrous ammonium sulphate, and separated from low molecular weight species via a PD10 desalting column (GE HEALTHCARE). Immediately desalted with 20 mM Tris-HCl pH 8, 150 mM NaCl and 5% (v/v) glycerol (wash buffer) using a PD10 column (GE HEALTHCARE), NIF5-catalysed in vitro cluster reconstitution was used to assemble FeS clusters on NBP35, as previously described (Crack et al., 2014). Briefly, NBP35 samples containing 70-90 μM protein in approximately 3.5 mL were treated with 3.75 mM dithiothreitol at 37 °C for 160 min. Reconstituted protein was then separated from low molecular weight species via a PD10 desalting column previously equilibrated with wash buffer.

UV-visible absorbance measurements were made with a JASCO V550 spectrophotometer. CD spectra were measured with a JASCO J810 spectropolarimeter. An extinction of 4406 nm = 16 000 M⁻¹ cm⁻¹ (Sweeney and Rabinowitz, 1980) was used to estimate the amount of Fe₅S₅ cluster present in NBP35 samples. Cluster stability was investigated under pseudo-first order conditions (~120 μM O₂) by combining varying aliquots of aerobic and anaerobic wash buffer (2 mL total volume) with NBP35 (~16 μM Fe₅S₅). Changes in the absorbance at 406 nm were used to track cluster stability.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Quantification of NPB35 protein levels in knockdown lines.

Figure S2. Alignment of Saccharomyces cerevisiae and Arabidopsis thaliana NBP35.

Figure S3. Root growth and transcript levels of FeS proteins in NBP35-C14A.

Figure S4. Developmental defects in plants expressing NBP35: NBP35-C14A.

Figure S5. Expression and purification of NBP35 and NBP35-C14A.

Figure S6. Spectroscopy of reconstituted NBP35 and NBP35-C14A.

Figure S7. NBP35 and DRE2 gene expression during development.

Figure S8. Confirmation of yeast-two-hybrid interactions identified in the screen.

Figure S9. Model of the CIA pathway in plants.

Table S1. Yeast-two-hybrid interactions of Arabidopsis NBP35 screened against a fragment library of seedling cDNA.

Table S2. List of primers.

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