Plastidic redox switches: Ferredoxins as novel RNA-binding proteins

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Ferredoxins (Fds) are small iron-sulfur proteins, which mediate electron transfer in a wide range of metabolic reactions. Several intriguing observations suggest that Fds may also directly associate with RNA, thus implicating a second role for these proteins in organelar RNA metabolism. Plants contain several closely-related Fd homologs, whose members are predicted to reside within the plastids. As strong mobile electron-carriers, able to partition between the stroma and the thylakoid membranes, Fds are therefore excellent candidates to regulate the expression of plastidic genes in a redox-dependent manner. Accordingly, the translation of D1 protein in the chloroplasts is mediated by a redox-poise involving the ferredoxin-thioredoxin system. Yet, despite these suggestive evidences, RNA binding activity has not been reported for an isolated Fd protein. Here, we established the intracellular locations of the six Fd paralogs in Arabidopsis to the plastids and demonstrated that one of these proteins, AtFd6, is associated with organelar transcripts in vivo. Biochemical analyses in vitro indicated that a re-combinant purified AtFd6-His protein binds with high affinity and specificity to \textit{psbA} mRNA, in a redox-dependent manner.

Keywords: ferredoxin, RNA-binding, translation, redox, chloroplasts, plants

Abbreviations: b-ME, 2-Mercaptoethanol; BSA, bovine serum albumin; CRM, Chloroplast RNA maturation domain; Cyt c, cytochrome c; EST, expressed sequence tags; Fd, ferredoxin; FNR, FD:NADP oxidoreductase; ORF, open reading frame; PS, Photosystem; RbcL, Rubisco large subunit; RBS, ribosome binding site; RRM, RNA recognition motif.

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Introduction

Ferredoxins (Fds) are small (10–16 kDa) iron-sulfur [2Fe-2S] proteins which are widely distributed in nature and mediate electron transfer in a large range of metabolic reactions (Fukuyama 2004). In photosynthesis, Fds carry a pivotal redox function throughout their essential role in transferring electrons from photosystem-I (PS-I) to Fd:NADP oxidoreductase (FNR, EC 1.18.1.2) (Arnon 1988). Yet, different isoforms are also present in non-photosynthetic cells, indicating that Fds regulate diverse physiological functions (Benz et al. 2010; Schröter et al. 2010; Winkler et al. 2010).

Circumstantial evidence suggest that Fds may directly associate with RNA, thus, implicating a second role for these proteins in the regulation of genome expression. In the SCOP database (Murzin et al. 1995) Fds share a common topology (the "ferredoxin-like" fold) of a basic charged β-surface supported by α-helices (Figure S1A) with known RNA-binding motifs; these including the RNA recognition motif (RRM), the 30/40 kDa subunit of RNA polymerases (Rodriguez-Monge et al. 1998), the CRM domain, a novel plant RNA-binding domain of ancient origins (Barkan et al. 2007; Keren et al. 2008), and the N-termini region of Rubisco’s large subunit (RbcL), which was recently shown to associate with its own transcript in a redox-dependent manner (Cohen et al. 2006). Moreover, in bacteria Fds are associated with nucleic acids \textit{in vivo} (Arendsden et al. 1995). As strong mobile electron-carriers, able to partition between the stroma and the thylakoid membranes, Fds are therefore excellent candidates to regulate the plastid genome expression in a redox-dependent manner. Accordingly, the translation of D1 protein is regulated by the redox-poise, involving the ferredoxin-thioredoxin system (Trebitsh and Danon 2001; Trebitsh et al. 2001; Levitan et al. 2005). However, it remains unknown whether Fds are directly associated with RNAs \textit{in vivo}.

The nuclear genomes of algae and plants harbor several Fd homologs, whose members are differentially expressed during different stages of development and under various environmental stimuli (Matsumura et al. 1997; Yonekura-Sakakibara et al. 2000; Hanke and Hase 2008). Arabidopsis contains six Fd paralogs (Table 1, Figure S1B; Hanke et al. 2004; Voss et al. 2010): AtFd1 (At1g10960) and AtFd2 (At1g60950) are closely related to one another and share high similarity with 'leaf-type' Fds (Hanke et al. 2004; Hanke and Hase 2008), whereas AtFd3 (At2g27510) encode to a 'root-type' isoform (Hanke et al. 2004). The forth paralog, AtFd4 (At5g10000), has several distinctive features and contains a highly degenerated catalytic site (Hanke et al. 2004). AtFd5 (also annotated as Fdc1; At4g14890) contains a short C-termini extension (Figure S1B) and was recently suggested to function in photosynthetic electron transport from PSI to NADP\textsuperscript{+} under high light conditions and/or limited availability of Fd1 or Fd2 proteins (Voss et al. 2010).
No data exists for Fd6 protein (At1g32550), which is well conserved in algae and plants (see Figure S2). Among the unique characteristics of Fd6 orthologs are the presence of a long carboxy-termini extension (Figures S1 and S2, and Hanke et al. 2004; Voss et al. 2010), and an additional cysteine residue within the Fe-S cluster (Figures S1 and S2). Multiple sequence alignments indicated that an analogous C-termini extension is also present within cyanobacteria, the proposed progenitors of plants plastids, suggesting ancient evolutionary origins for this region within Fd6 proteins.

To examine the putative roles of Fds in plastidic RNA metabolism, we established the intracellular locations of the six Fd paralogs in Arabidopsis and analyzed the electron transport activity and RNA-binding characteristics of two distinct members of this family, AtFd1 and AtFd6 proteins. In agreement with their predicted localizations (Table 1) and immunoblot analyses of several members of this family (Hanke et al. 2004), transient expression assays with a green fluorescent protein (GFP) tag localized the six Fd paralogs in Arabidopsis to the plastids. Fractionation of a green fluorescent protein (GFP) tag localized the six Fd proteins.

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Material and Methods

Protoplast preparation and GFP-based transient assay

Fragments corresponding to the N-termini of each Fd (Figure S1B) were fused in-frame to GFP by cloning each fragment with Ncol and XhoI sites into pTEX-GFP vector (gift of Dr. Yoram Eyal; ARO). Specific primers used in the reactions are listed in supplementary Table S1. Mesophyll protoplasts were isolated from tobacco (Nicotiana tabacum cv Samsun NN) plants grown under sterile conditions (Drap et al. 1988). Electroporation of ~5x10^5 protoplasts per et al. 1988). Electroporation of ~5x10^5 protoplasts per cuvett (Novagen), expressed in BL-21 star cells; Stratagene) as recombinant proteins containing a C-termini 6xHis tag and purified by immobilized-metal affinity chromatography (Ni-NTA beads, Adar-Biotech, IL). The purified proteins were then dialyzed against 50% (v/v) glycercol buffer (50 mM HEPES-KOH, 500 mM KCl, 0.1% Triton X-100, 5 mM [Mg, pH 7.0], aliquoted into 200 µl fractions and stored at -20°C. Under these conditions the proteins retained their full activity for few days, during which the binding experiments were performed. SDS-PAGE analyses of AtFd1 and AtFd6 proteins were in good agreement with the expected sizes (11 kDa and 16 kDa, respectively) and confirmed that the purity of the eluted protein was high (> 95%) (supplementary Figure S3). The integrity of the trap

Protein extraction and analysis

Arabidopsis thaliana (ecotype Columbia) was used in all experiments. Total leaf protein was extracted from 3-weeks-old leaves by a borate/ammonium acetate method (Maayan et al. 2008). Protein concentration was determined by the Bradford method (BioRad) according to the manufacturer's protocol, with BSA used as a calibrator. Total leaf and organellar proteins were mixed with 3x Laemmli sample loading buffer (Laemmli 1970) and subjected to SDS-PAGE, at a constant 100 V. Following electrophoresis, the proteins were visualized with Coomassie blue stain, or transferred to a nitrocellulose membrane (Whatman) and blotted overnight at 4°C with specific primary antibodies. Detection was then carried out by chemiluminescence assay after incubation with an appropriate horse-radish peroxidase (HRP)-conjugated secondary antibody (Sigma or Santa Cruz).

Chloroplast isolation, sucrose gradients fractionations and RNA destabilization experiments

Intact chloroplasts were isolated from 3-weeks-old Arabidopsis leaves by percoll gradients (Kunst 1998). Chloroplast lysates were prepared from intact chloroplasts by resuspending chloroplast pellets in 10 mM Hepes-KOH (pH 8), 10 mM MgCl2 buffer. Thylakoids were obtained by centrifugation at 3,200 g for 10 min at 4°C, while stromal extract was prepared from the resulting supernatant by centrifugation at 42,000 g for 30 min at 4°C. Mitochondria were isolated from cauliflower (Brassica oleracea) inflorescences, as previously described (Keren et al. 2009). For the analysis of native organellar complexes, stroma was fractionated by sucrose gradient (10 to 50%) centrifugation at 157,000 g for 4 hours at 4°C. Fractions (top to bottom) were then collected and analyzed by immunobassays. For RNA destabilization experiments (see Uyttewaal et al. 2008), the stroma was treated with 40 U/ml RNAse-inhibitor (Fermentas), 10 U/ml RNase-free DNase (Promega) or 1.0 µg/ml micrococal ribonuclease (Sigma), prior to its loading on sucrose gradients.

Expression and purification of recombinant Arabidopsis Fd1 and Fd6 proteins

Fragments corresponding to AtFd1 and AtFd6 genes were generated by RT-PCR (specific oligonucleotides are listed in Table S2); the cDNAs were cloned into pET28a plasmid (Novagen), expressed in E. coli cells (BL-21 star cells; Stratagene) as recombinant proteins containing a C-termini 6xHis tag and purified by immobilized-metal affinity chromatography (Ni-NTA beads, Adar-Biotech, IL). The purified proteins were then dialyzed against 50% (v/v) glycercol buffer (50 mM HEPES-KOH, 500 mM KCl, 0.1% Triton X-100, 5 mM [Mg, pH 7.0], aliquoted into 200 µl fractions and stored at -20°C. Under these conditions the proteins retained their full activity for few days, during which the binding experiments were performed. SDS-PAGE analyses of AtFd1 and AtFd6 proteins were in good agreement with the expected sizes (11 kDa and 16 kDa, respectively) and confirmed that the purity of the eluted protein was high (> 95%) (supplementary Figure S3). The integrity of the trap

Antisera

Antibody to AtFd6 protein (Rimon Biotech, IL) was generated against two synthetic peptides corresponding to the carboxy-termini region of AtFd6 protein (Figure S1B). Polyclonal antibodies to AtFd1, ATP-synthese β-subunit (AtpB), cytochrome c oxidase subunit 2 (COX2), and D1 protein (PsbA), were purchased from Agrisera. Antibodies to PsbO (33 kDa subunit of the oxygen evolving complex) and Rubisco were generously donated by Prof. Zach Adam (Hebrew University) and Prof. Michal Shapira (Ben Gurion University), respectively.
proteins was verified using electrospray ionization ion mass-spectrometry (The Smoler Proteomic Center, Technion, IL).

**Assay of Fd-linked enzyme activity**

The activity of recombinant AtFd1-His and AtFd6-His proteins was determined on the basis of their ability to mediate electron transfer in photochemical reduction of cytochrome c (Cyt c) by thylakoids or by electron transfer from HADPH-reduced FNR to Fd (Hase et al. 1991). Photo-reduction of NADP+ (Sigma) was measured by the absorbance change at 340 nm upon illumination of thylakoids (10 µg/ml) in buffer containing 50 mM HEPES-KOH pH 8.0, 100 mM NaCl, 1 mM MgCl2 and the desired amount of recombinant AtFd 1 and 6 proteins. Cyt c (Sigma) reduction by FNR (Sigma, CAS no. 9029-33-8) was determined in 1 ml buffer containing 50 mM Tris-HCl (pH 7.5), 50 µM oxidized horse Cyt c (Sigma), 100 µM NADPH (Sigma) and various concentrations of recombinant AtFd 1 and 6 proteins, by the increase of absorbance at 550 nm.

**In vitro transcription of RNA templates**

RNAs used for binding studies were obtained by RT-PCR with forward DNA oligonucleotides containing the consensus T7 promoter site upstream to each fragment (see supplementary Table S3). In vitro transcription of ‘body-labeled’ RNAs was carried out by T7 RNA polymerase (Promega) with 0.5 mM ATP, GTP, and CTP and 50 µM UTP nucleotides in the presence of 20 µCi [α32P]-UTP (3,000 Ci/mmol). Contaminating DNAs were removed by DNase treatment (Promega), and the in vitro transcribed RNAs were purified by using the RNasy kit (Qiagen), according to the manufacturer’s directions. RNA was visualized by electrophoresis of a 1/100 dilution of each transcript on a denaturing (7 M urea) polyacrylamide gel, which was subsequently dried down and autoradiographed.

**RNA-binding assays**

Filter-binding assays were performed essentially as previously described (Östergeset et al. 2005; Keren et al. 2008). In vitro transcribed RNAs were renatured by heating to 95°C and the RNA was then allowed to refold in the presence of 10 mM MgCl2. Binding reactions (20 µl) contained 25 pM renatured RNAs in reaction buffer containing 5 mM DTT, 10 µg/ml BSA and 1 U/µl RNase inhibitor. Protein was added to the reaction mixture and incubated for 15 min at 25°C to allow Fd-RNA complex formation. The reactions were chilled on ice and were then passed through a sandwich of nitrocellulose and charged nylon membranes by vacuum filtration, using a slot-blot manifold (Gibco-BRL). The membranes were washed twice with 200 µl reaction buffer, dried for 5 min at room temperature and exposed to PhosphorImager screen (Fuji). Data were quantified by ImageQuant software (Version 5.1; Molecular Dynamics) and the fraction of bound RNA was calculated as the ratio between RNA captured by the nitrocellulose membrane and the total RNA signals by both membranes.

**‘In silico’ analyses**

Multiple alignments were done with ClustalX (Jeanmougin et al. 1998). Predicted 3D structures of individual Fd domains were constructed by homology modeling using the Swiss-Model server (Schwede et al. 2003), with the established spinach Fd1 structure (PDB 1A70). The visualization and manipulation of obtained models were arranged by the PyMol package (http://www.pymol.org).

**Results**

The six Fd paralogs in Arabidopsis are localized to the plastids

The nuclear genome in Arabidopsis (The Arabidopsis Genome Initiative 2000) harbor six genes that are closely related to “model” Fds (Table 1; Hanke et al. 2004; Voss et al. 2010). Analysis of their expression profiles, available in EST databases and the ‘Genevestigator microarray database and analysis toolbox’ (Zimmermann et al. 2004), suggested that they are differentially expressed in a tissue-dependent manner (data not shown). While AtFd2 demonstrated the highest expression levels, the lowest signal was observed in
the case of AtFd6. The mRNA levels of AtFd 1, 2, 5 and 6 peaked in vegetative tissues, while the expression AtFd3 was higher in the roots. These data are in good correlation with the protein levels of Fd 1, 2 and 3, as indicated by immunoassays with Arabidopsis leaves and roots extracts (Hanke et al. 2004). Although immunoassays suggested that AtFd4 is accumulating to low levels in the leaves (Hanke et al. 2004), its expression could not be supported by ESTs (NCBI) or microarray (Genevestigator) databases, nor by RT-PCR analysis (data not shown).

Targeting prediction programs suggest that the six Fds paralogs in Arabidopsis, as well as their rice orthologs, are all localized to the plastids (Table 1). Yet, despite their predictive power these programs are still inaccurate, ascribing many non-plastidic proteins to that organelle (Heazlewood et al. 2005). To establish their intracellular locations in vivo, constructs encoding the N-terminal regions (see Figure S1B) of each Arabidopsis Fd paralog was cloned in-frame to GFP, introduced into tobacco protoplasts and the location of each GFP-fusion protein was determined by confocal microscopy.

While the signals of plastidic Rubisco small subunit (RbcS) and the mitochondrial ATP synthase β-subunit (AtpB) co-localized with those of chlorophyll autofluorescence and MitoTracker marker (a mitochondrion-specific fluorescent probe), respectively, the signal of the dualy-localized cysteinyl-tRNA synthetase (CysRS) protein (Peters and Small 2001) was observed in both chloroplasts and mitochondria (Figure 1). When tobacco protoplasts were transfected with the N-termini regions of the six AtFd paralogs fused to GFP, the signals were exclusively detected as orbicular discs, co-localizing with those of chlorophyll autofluorescence (Figure 1). Clear localization of the different Fds-GFPs to the chloroplast was also obtained with Arabidopsis protoplasts (data not shown).

To more carefully establish the localizations of AtFd1 and AtFd6 to the plastids, we performed immunoblot analysis of Arabidopsis organelar extracts, with antibodies raised against different organelar proteins. As anticipated by their ‘in silico’ organellar predictions (Table 1) and GFP localizations (Figure 1), the anti-AtFd 1 and 6 antibodies cross-reacted with plastidic proteins in the expected sizes for mature AtFd1 and AtFd6 proteins (i.e. about 10 and 16 kDa, respectively) (Figure 2). Immunoassays with the plastidic PsbA (D1 protein) and PsbO (33 kDa subunit of the oxygen evolving complex) proteins and the mitochondrial COX2 (cytochrome c oxidase subunit 2) protein confirmed that the organelle preparations were reasonably pure (Figure 2); however, due to their cross-reactivity, the anti-AtpB (ATP synthase β-subunit) antibodies cross-reacted with AtpB subunits in both the plastid and mitochondrial fractions (Figure 2).

For organellar subfractions, chloroplast lysates were obtained from intact chloroplasts by resuspending the chloroplast pellets in buffer containing 10 mM Hepes-KOH (pH 8), 150 mM KCl, 10 mM MgCl2; the membranous fraction containing the thylakoids was obtained by centrifugation at 3,200g (10 min at 4°C), while the stroma was prepared from the resulting supernatant by a sequential centrifugation at 42,000g (30 min at 4°C). Under these conditions, AtFd1 and AtFd6 proteins were distributed between the stroma and thylakoid fractions, whereas the signal of AtFd2 was observed mainly within the stroma (Figure 2).

**AtFd6 is found in RNA-associated particles in vivo**

To analyze whether AtFd1 and AtFd6 proteins are associated with organellar transcripts in vivo, about 2.0 mg stromal protein was fractionated by velocity centrifugation sedimentation throughout sucrose gradients and each fraction was analyzed by immunoassays with anti-AtFd 1 and 6 antibodies. For RNase-sensitivity experiments, the stroma was treated with RNase-inhibitor (as control), RNase-T1, RNase-free (+DNase), or ribonuclease A (+RNase A), prior to its loading on the sucrose gradients. As indicated in Figure 3, AtFd1 and AtFd6 particles migrated throughout the sucrose gradients with calculated molecular masses ranging from 50 to 250 kDa, significantly higher than their expected monomeric sizes (10 and 16 kDa, respectively). While the mobility of AtFd6 particles substantially decreased upon the RNase treatment, the sedimentation profiles of AtFd1 and AtFd2 particles was found to be less sensitive to the RNase treatment. The native Fd6 complexes were affected to only a small degree by the DNase treatments. Nuclease treatment of the stroma had a minor effect on the sedimentations of Rubisco and ClpC complexes (Figure 3). These results indicated that AtFd6 is found in a stable ribonucleoprotein (RNP) particle(s) within the stroma.
Figure 1: Analysis of the intracellular localization of the Arabidopsis Fd protein family. Tobacco protoplasts were transformed with constructs containing the N-termini regions of Rubisco small subunit (RbcS), ATP synthase β-subunit (AtpB), cysteinyl-tRNA synthetase (CysRS), and the six Fd paralogs in Arabidopsis (see Figure S1) fused in-frame to GFP. GFP signals (green, upper left side), chlorophyll autofluorescence (blue, lower left side), 0.4 µM MitoTracker marker (red, upper right side) and merged images (lower right side) are outlined in each panel.

Figure 2: Fd1 and 6 are localized to the chloroplasts in vivo. Immunoblots of 50 µg protein of intact chloroplasts (CLPs), thylakoids (TLK) and stromal (STR) subfractions. Mitochondria (Mit) were obtained from cauliflower inflorescences as previously described (Keren et al. 2009). The blots were probed with polyclonal antibodies raised against Fd1 and Fd6 proteins, ATP synthase β-subunit (AtpB), photosystem-II D1 protein (PsbA), the 33-kDa subunit of the oxygen evolving complex (PsbO), and the mitochondrial cytochrome oxidase subunit 2 (COX2). Detection was carried out by chemiluminescence assay after incubation with HRP-conjugated secondary antibody.
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Figure 3: AtFd6 is associated with organellar transcripts in vivo. Stromal protein preparations were treated with RNase inhibitor (control), RQ1 DNase (RNase-free) or DNase-free ribonuclease (+RNase) and fractionated by sucrose gradient ultracentrifugations. Aliquots of total organellar extracts (Str) and sucrose gradient fractions were subjected to immunoblot analyses with different antibodies, as indicated in each blots. High molecular mass standards sizes are indicated above the blots and are given in kilodaltons. The calculated size of AtFd1 is ~11 kDa; AtFd6, ~16 kDa; Rubisco large subunit (RbcL), ~55 kDa; and ~80 kDa for the plastidic ClpC protease.

In vitro enzymatic and RNA-binding activities of Arabidopsis Fds

To establish whether Fds are directly associated with RNAs, AtFd1 and AtFd6 were expressed as recombinant proteins containing a 6x His tag on their C-termini, purified and were then analyzed for their associated electron-transport and RNA-binding activities (Figure S3).

Electron transport activity was measured by illuminating thylakoid membranes in the presence of Cyt c and recombinant AtFd 1 and 6 proteins. These assays indicated that AtFd6 is capable of receiving electrons from PS-I, although demonstrated lower affinity for electron transferred from PS-I than that of AtFd1-His (Figure 4A). FNR catalyzes the transfer of electrons from NADPH to Cyt c in the presence of Fds (Hase et al. 1991). As indicated in figure 4B, both AtFd1-His and AtFd6-His proteins were able to mediate electron transfer between NADPH and Cyt c; yet, the mean Cyt c reduction activity of AtFd6-His protein was significantly lower than that of AtFd1-His (Figure 4B). These results suggested that AtFd6 is able to function as electron-carrier within the plants chloroplasts.

RNA-binding activity assays were performed by incubation of 32P-labeled RNA templates (25 pM) with purified AtFd 1 and 6 proteins for 15 min at 25°C in a 20 µl reaction mix containing 10 µg/ml BSA and 1 U/µl RNase inhibitor. The reactions were then transferred to ice and the binding was examined by filtration of the 'RNA-protein' complex through a "sandwich" of nitrocellulose (for capturing RNP particles) and charged-nylon filters (for capturing unbound RNAs) (see supplementary Figure S4A). The membranes were washed twice with 200 µl of reaction buffer, dried and quantified with a PhosphorImager. The fraction of 'RNA-
bound' was calculated from the ratio between the signal from the nitrocellulose-captured RNA and the total RNA signals obtained by both membranes.

The translation of the D1 protein is regulated by the redox poise of the ferredoxin-thioredoxin system (Trebitsh and Danon 2001; Trebitsh et al. 2001; Levitan et al. 2005). This regulation is mediated by a set of proteins, whose identity is partially known, which bind to the 5′-UTR of psbA mRNA. Preliminary binding results indicated that AtFd6-His protein binds to psbA mRNA in vitro. Optimal binding occurred at pH 7.0, 50 mM K⁺ and 20 mM Mg²⁺ (Figure 4C and D). Yet, AtFd1-His demonstrates only low affinities (in the low mM range) in its binding to various RNAs under many different binding conditions tested (Figure S4 and data not shown). In the presence of 20 mM MgCl₂ the affinity of AtFd6-His to psbA mRNA decreases as the [K⁺] was increased above ~50 mM (Figure 4C, left pane). This "salting off" effect may indicate that electrostatic interactions contribute to the binding stability of AtFd6-RNA interactions. The binding is strongly dependent upon [Mg²⁺] in binding reactions containing 50 mM [K⁺] (Figure 4D, right panel), suggesting that optimal binding might also involve a Mg²⁺-dependent structures within the RNA molecules. When AtFd6-His protein was preheated to a temperature above 65°C the apparent RNA binding activity dropped precipitously (data not shown), indicating that a native conformation is required for AtFd6 binding activity. Equilibrium binding assays were performed by the incubation of 25 pM (³²P)-labeled RNA with increased concentrations (1-1,000 nM) of AtFd6 protein for 15 min at 25°C in a 20 μl reaction mix (50 mM Tris-HCl pH 7.0, 50 mM KCl, 20mM MgCl₂, 5 mM DTT, 10 µg/ml BSA, 1 U/µl RNase inhibitor). By fitting the binding data with the Hill model (ORIGIN 7.5 software; Microcal Software Inc.), the calculated dissociation constant value (K₀) for AtFd6-His binding.
Figure 5: AtFd6 binds with high affinity and some specificity to psbA mRNA in vitro. Equilibrium binding assays were performed by the incubation of increasing amounts of recombinant purified Fd proteins with different plastidic 25 pM (32P) -labeled RNAs, in standard binding conditions (i.e. 50 mM Tris-HCl pH 7.0, 50 mM KCl, 20 mM MgCl₂, 5 mM DTT, 10 µg/ml BSA, 1 U/µl RNase inhibitor) without (A) or in the presence (B) of 50 µg/ml yeast tRNA, as competitor RNA. The reactions were transferred to ice and the binding was assayed by filtration of the RNA/protein complex throughout a nitrocellulose / charged-nylon filters sandwich. The fraction of RNA bound was calculated by the relative amount of 32P signal on the nitrocellulose and Nylon-charged membranes from at least five independent assays. Dissociation constants \( K_D \) were determined by using ORIGIN 7.5 software (Microcal Software Inc., Northampton, MA).

The dissociation constant \( K_D \) to psbA mRNA was 5.3 ± 3.1 nM, with apparent Hill coefficient \( n_H \) of 0.9 ± 0.4 (Figure 5A), suggesting a non-cooperative binding activity. No, or very low, activity was observed in the absence of either DTT or 2-mercaptoethanol [β-ME] in the reaction mix (Figure 5A), suggesting that AtFd6 binds to psbA mRNA in a redox-dependent manner.
We further assayed the binding specificities associated with AtFd6-His protein to different RNAs (Figure 5); these including the β-subunit of acetyl CoA (accD), cytochrome c biogenesis factor A (ccsA), Rubisco's large subunit (rbcL), RNA polymerase subunit A (rpoA), psbD, and the antenna PS-II D1 (psbA) and D2 (psbD) transcripts. Under optimal binding conditions (i.e. 50 mM K+, 20mM Mg+2, pH 7.0) AtFd6-His associated with similar affinities to all RNAs (K D's ranging from 5 ~ 35 nM) (Figure 5A and data not shown). Yet, when tRNA (50 µg/ml) was added to the reaction mix, AtFd6-His demonstrated a higher specificity in its binding to psbA mRNA (Figure 5B). While the K value of AtFd6-His to psbA mRNA was about 30 nM (Figure 5B) in the presence of the tRNA, its calculated dissociation constants to accD, ccsA, rbcL, rpoA and psbD were considerably lower (K D's ranging from 135 to 280 nM) (Figure 5B and data not shown). Very little binding was observed when the RNA was substituted from 135 to 280 nM) (Figure 5B and data not shown). Very little binding was observed when the RNA was substituted from 135 to 280 nM) (Figure 5B and data not shown). Very little binding was observed when the RNA was substituted from 135 to 280 nM) (Figure 5B and data not shown). Very little binding was observed when the RNA was substituted from 135 to 280 nM) (Figure 5B and data not shown). Very little binding was observed when the RNA was substituted from 135 to 280 nM) (Figure 5B and data not shown).

**AtFd6 binding to psbA mRNA is mediated through its association with the 5’-UTR**

In addition, we also assayed the binding specificities associated with AtFd6-His protein binding to several truncated fragments of psbA RNA; these including the intact psbA mRNA, psbA RNA lacking the 5’ and 3’ UTR sequences (psbA ΔUTR) and psbA Δ5’-UTR or psbA Δ3’-UTR (see Figure 6A). For this purpose, increasing amounts of AtFd6-His (1-1,000 nM) were incubated with the psbA transcripts (25 µM 32P-labeled RNAs) in reaction mix (i.e. 50 mM K+, 20mM Mg+2, pH 7.0) containing 50 µg/ml tRNA. As indicated in figure 6B, maximal binding of Fd6 to psbA RNA was observed with the intact psbA transcript. The formation of stable AtFd6-psbA RNP particles was substantially reduced when the protein was incubated with truncated psbA molecules, lacking the UTR sequences (psbA ΔUTR, K D ≈ 170 nM) (Figure 6B). Reduced binding activity was also observed with psbA Δ3’-UTR (K D ≈ 60 nM) and to a greater extent in the case of psbA lacking the 5’-UTR, where the association of AtFd6-His with psbA RNA was about 10 times lower (K D ≈ 207 nM) than that observed with the intact mRNA (Figure 6B). When the binding of AtFd6-His was assayed with individual RNA fragments corresponding to the 5’ or 3’ UTR sequences, AtFd6-His protein was found to bind to these RNAs with calculated K values at the low nM range (132 nM and 217 nM, respectively) (Figure 6B).

Taken together, these results indicated that AtFd6-His is able to bind to RNA in a redox-dependant manner, in vitro. In the presence of competitor RNA Fd6 demonstrated higher specificity in its binding to psbA RNA, in comparison with various other plastidic transcripts. AtFd6 binding to psbA is mediated through its association with the 5’ UTR, but also seems to form interactions with various regions found within the 3’-UTR and coding sequences.

**Discussion**

Chloroplasts, the green plant organelles in which photosynthesis takes place, contain their own genetic systems (cpDNA). The expression of the cpDNA is complex and requires extensive post-transcriptional modifications and a tight regulation of the translation of the mature transcripts (reviewed in Barkan 2004; Gagliardi and Binder 2007; Bonen 2008). These processes are subjected to developmental and environmental stimuli and are regulated by many different proteincous cofactors. The roles of RNA-mediated regulation and redox switches in these events are unambiguous but our understanding of such mechanisms remains quite limited.

Fds are small iron-sulfur proteins which mediate electron transfer in a wide range of biological reactions (Knaff 2005). Yet, several lines of evidence support the idea that Fds may directly associate with RNAs, thus implicating a second role for these proteins in gene expression: the 'ferredoxin-like' fold include RNA-binding proteins of species as diverse as _E. coli_ and _humans_ (Murzin et al. 1995). Moreover, in bacteria, Fds are associated with nucleic-acids in vivo (Thomson,1991; Arendsen et al. 1995). Arabidopsis contains six Fd paralogs (Table 1 and Figure S1) which are all localized to the plastids (Figure 1). Here, we analyzed the RNA-binding characteristics of two distinct members of this family, AtFd1 and AtFd6. While _AtFd1_ encodes a 'leaf-type' Fd, AtFd6 is more degenerated and contains a long C-termini extension that is speculated to surround the redox center (Figures S1 and S2; and Hanke et al. 2004; Voss et al. 2010). Immunooassays indicated that both AtFd 1 and 6 proteins are distributed between the stroma and thylakoid fractions (Figure 2). In vitro enzymatic activity assays demonstrated that AtFd6-His is able to receive electrons from PS-I, although the protein demonstrated lower activity than that of AtFd1 (Figures 4A and B). Sucrose gradient fractionation of stromal proteins suggested that AtFd6 is found in stable RNA-associated particles in vivo (Figure 3). Thus, the ability of Fd6 to mobile between the stroma and the thylakoids indicate a unique adeptness for regulating 'RNA-metabolism' and 'redox-dependent mechanisms. Yet, despite these suggestive evidences, RNA binding activity has not been reported for an isolated Fd protein.
To analyze whether Fds are directly associated with RNA, AtFd1 and AtFd6 were expressed in *E. coli* cells, purified and analyzed for their associated RNA-binding activities in vitro. While AtFd1 demonstrated low affinities in its binding to various RNAs (Figure S4 and data not shown), AtFd6 bound with high affinity to *psbA* mRNA (Figures 5 and 6). Low binding was observed in the absence of DTT or β-ME in the reaction mix (Figure 5A); these results may
indicate to conformational rearrangements within AtFd6 resulting from the oxidation state of various cysteine residues within the molecule. Accordingly, the interaction between FNR and Fd was shown to be mediated by redox-linked conformational changes of the Fd protein (Morales et al. 2000). The unique cysteine residue within Fd6 may contribute to this "redox regulation" of AtFd6 RNA-binding.

To examine the binding characteristics of AtFd6-psbA RNP complex the fraction of the bound psbA mRNA (Figure S4A) was plotted relative to the concentration of AtFd6-His. In 'standard' binding salts (i.e. pH 7.0, 50 mM K+, 20 mM Mg2+), a dissociation constant (Kd) of approximately 5 nM was estimated for the full-length mRNA (Figure S5A). The binding curve displays a Hill coefficient of ~1.0 (Figures 5A and 5B), indicating to non-cooperative binding. The affinity of AtFd6-His to psbA was then compared with a number of other RNA-protein interactions (Figure 5). Under these conditions AtFd6 bound with similar affinities to different plastidic transcripts (Figure 5A). Yet, the addition of tRNA revealed AtFd6 specificity for psbA mRNA (Figure 5B). The reduced amount (~50%) of bound psbA mRNA (Figure 5) indicates that the binding sites are inaccessible in about half of the molecules, possibly due to misfolding of the in vitro transcribed psbA RNA, which is likely to fall into stable non-native structures (Silverman et al. 2006).

A series of truncations were made to determine which regions of psbA mRNA are responsible for Fd6-psbA RNP formation (Figure 6A). For each construct, a Kd and nH were determined. The truncated RNAs yield only partial RNP formation (Figure 6B), in which the 5'-UTR region appeared to have the largest impact on the formation of stable Fd6-psbA particles (Figure 6B); altered Hill coefficient values (nH ≥ 2.0) for the truncated psbA transcripts lacking the 5'-UTR (psbA Δ5UTR and psbA Δ5UTR, Figure 6A) indicate a disruption of RNA-protein interactions, and suggested that the 5'-UTR region includes important protein-binding sites that are necessary for the association of Fd6 with psbA mRNA.

In summary, our current understanding of post-transcriptional regulation of gene expression by redox active proteins is still in its infancy, with many basic questions remaining unanswered (Danon 2002; Tang et al. 2003). The list of newly identified redox regulated proteins and biological-pathways is rapidly expanding, but it is yet mostly unclear how redox signals are transduced and perceived intracellularly. Not less importantly, new fundamental roles in post-transcriptional regulation of gene expression are being identified for RNAs and their interacting proteins partners.

Our data indicated that AtFd6 carries two distinct biological functions: electron transport and binding to psbA mRNA. The importance of translational control sequences within the 5'-leader sequences was demonstrated for many plastidic transcripts (Marín-Navarro et al. 2007). It remains possible, therefore, that the association of Fd6 with the 5'-UTR of psbA may induce or stabilize RNA conformational changes (see Kozak 2005) required for the efficient translation of D1 protein. However, a more comprehensive analysis is required to determine whether the binding of AtFd6 affects the folding of psbA and ultimately its translation within the chloroplasts upon illumination.

Recently, Cyt c was shown to interact with tRNA in the mitochondria and cytosol (Mei et al. 2010). Interestingly, both Fd6 and Cyt c are mobile electron carriers which contain iron-sulfur clusters within their active sites; these were apparently evolve early in the evolution of life (Eck and Dayhoff 1966; Goodsell 2004). As putative reminiscence of an ancient 'RNA world' (Gesteland et al. 1999), these proteins may have therefore retained their "RNA-binding" activities as means to coordinate genome expression and organellar functions. Regardless of their evolutionary origins, it would be interesting to test whether additional proteins of the electron-transfer machineries also carry similar "moonlighting" (see Jeffer 2003) RNA-binding activities.

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References


Ferredoxins as plastidic redox switches, Kolton M et al.

Supplementary Figure S1: (A) Spinach ferredoxin-1 structure (PDB: 1A70) illustrated as either ribbon (left panel) or by potential electrostatic surface (right panel), showing basic charged $\beta$-surfaces supported by an $\alpha$-helix. (B) Sequence alignment of Arabidopsis ferredoxin family. An alignment of the predicted Fd amino-acid sequences was generated in ClustalW 1.8. Transit peptides are shown in green box, which were estimated by comparison with known mature Fd proteins according to Hanke et al. (2004). White residues on black background are those conserved in all six sequences analyzed. The [2Fe-2S] cluster typical to plant-type Fds is highlighted by a blue box, while the conserved four cysteine residues of the Fd superfamily, involved in the [2Fe-2S] cluster binding are indicated by red asterisks. The blue arrow points to the unique cysteine residue (C-100) in AtFd6. The two synthetic peptides used for the generation of antibodies against AtFd6 are underlined (red lines) on AtFd6 sequence.
Supplementary Figure S2: Sequence alignment Fd6 orthologs in various plants and algae. An alignment of the Fd6 amino-acid sequences was generated in Clustal W 1.8. The putative transit peptides, the [2Fe-2S] cluster and the unusual C-termini extension regions are marked by black arrows. The unique cysteine residue within the Fe-S cluster in AtFd6 (C-100) is marked by a blue arrow. *Arabidopsis thaliana*, NP_174533; *Chlorella variabilis*, EFNS2397; *Cyanobacteria sp. PCC 7822*, YP_003886341; *Physcomitrella patens*, XP_001768017; *Picea sitchensis*, ACN40449; *Populus trichocarpa*, XP_002329520; *Oryza sativa*, NP_001050923; *Ricinus communis*, XP_002519835; *Vitis vinifera*, XP_002281131; *Volvox carteri*, XP_002946915; *Zea mays*, ACG28100.
Supplementary Figure S3: Expression and purification of recombinant AtFd1-His and AtFd6-His proteins. Fd 1 and 6 proteins, lacking their predicted N-terminal chloroplast targeting presequences (mature proteins), were expressed as a fusion protein containing C-termini 6xHis tag. The cells were ruptured by passage twice through a French pressure cell (20,000 lb/in²), and following centrifugation of the resulting lysate (~27,000 xg for 30 min at 4°C); the proteins were affinity purified from the resulting supernatant by Ni-NTA chromatography (FPLC apparatus). The protein profiles of non-induced (UI), E. coli cells induced with 1 mM IPTG for 3 hours (Ind), supernatant (S), unbound proteins (UB) and fractions obtained from the column were analyzed by SDS-PAGE. Fractions that contained the proteins were combined and dialyzed against 50% (v/v) glycerol buffer (i.e. 50 mM HEPES-KOH, 500 mM KCl, 0.1% Triton X-100, 5 mM βME, pH 7.0), aliquoted into 200 µl fractions and stored at -20°C.
Supplementary Figure S4: Ferredoxin binding activities. Equilibrium binding assays were performed by the incubation of 25 pM $^{32}$P-labeled psbA RNA with increased concentrations of purified His-tagged AtFd1 and AtFd6 proteins (1 – 1000 nM) for 15 min at 25°C in a 20 µl reactions mix containing 50 mM KCl, 20 mM Mg$^{2+}$, 5 mM DTT (pH 7.0) in the presence of 10 µg/ml BSA and 1 U/µl RNase inhibitor. The reactions were transferred to ice and the binding was assayed by filtration of the RNA/protein complex throughout a nitrocellulose/charged-nylon filters sandwich (A). Protein-RNA complexes were retained on the nitrocellulose membrane ("bound"); RNA that passed through the nitrocellulose was retained on the nylon membrane underlay ("free"). Raw slot blot data is shown from a representative experiment. The fraction of RNA bound was calculated by the relative amount of $^{32}$P-signal on the nitrocellulose and Nylon-charged membranes (B). The values represent the means of five experiments with three different batches of purified proteins, while the Y-axis bars represent the SD of individual experiments.
## Supplementary Table S1. DNA oligonucleotides used in GFP localization analysis.

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
</table>
| AFD1       | Forward: AA ACC ATG CTC ACT CTC TCA  
             Reverse: AAA CTC GAG AGC ACA GCT CGA ACA AGA ACC |
| AFD2       | Forward: AAA CTC ATG CTC ACT CTC TCC  
             Reverse: AAA CTC GAG GGC ACA AGT TGA CCA AGA ACC |
| AFD3       | Forward: AAA CTC ATG GCG ACT GTA CGA ATC TCC  
             Reverse: AAA CTC GAG GGC TGC GCA AGA GTA AGG CAA |
| AFD4       | Forward: AAA CTC ATG GAT UAA GTA CTC TAC TCC  
             Reverse: AAA CTC GAG ACA ACA TGT CCC ACA AGT CCC |
| AFD5       | Forward: AAA CTC ATG CTC CGT ATT TGC CCC TGT  
             Reverse: AAA CTC GAG ACC AGT CCT GCA AGA AAC GG |
| AFD6       | Forward: AAA CTC ATG GCG ACT CTC CTC CGG ACT  
             Reverse: AAA CTC GAG TTT TGC TGC GCA AGT CAT ACA AAC |
| N. sylvestris ATP synthase β subunit   |
|            | Forward: TATACAATGCTCCTCGAGGG  
             Reverse: TCTCTGTGAAGACATTAGGCGGCTTAGTAAAA |

(1) Nicotiana sylvestris nuclear-encoded mitochondrial ATP synthase β subunit.

## Supplementary Table S2. DNA oligonucleotides used for the construction of C-termini 6xHIs taggged Fd proteins in pET28a plasmid.

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
</table>
| mAFD1-His  | Forward: 5’ - AAAACATAGCTGCTAAATCAAGTCAAGTCCATC  
             Reverse: 5’ - TTCTGGAACATAGGCTCTCTCTCTGT |
| mAFD6-His  | Forward: 5’ - AAACATAGCTGCTAAATCAAGTCAAGTCCATC  
             Reverse: 5’ - TTCTGGAACATAGGCTCTCTCTCTGT |

## Supplementary Table S3. DNA oligonucleotides used for the construction of plastidic DNA templates under the control of the T7 RNA polymerase consensus site.

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
</table>
| phdF Full (a) | Forward: 5’ - TAATAAGACTCACTATAAGGATAAAGCTCTCAATACT (a)  
             Reverse: 5’ - TCTCAATAAAGGATAAAGCTCTCAATACT |
| phdF | Forward: 5’ - TAATAAGACTCACTATAAGGATAAAGCTCTCAATACT  
             Reverse: 5’ - TCTCAATAAAGGATAAAGCTCTCAATACT |
| phdA | Forward: 5’ - TAATAAGACTCACTATAAGGATAAAGCTCTCAATACT  
             Reverse: 5’ - TCTCAATAAAGGATAAAGCTCTCAATACT |
| phdA-5’UTR | Forward: 5’ - TAATAAGACTCACTATAAGGATAAAGCTCTCAATACT  
             Reverse: 5’ - TCTCAATAAAGGATAAAGCTCTCAATACT |
| phdA-3’UTR | Forward: 5’ - TAATAAGACTCACTATAAGGATAAAGCTCTCAATACT  
             Reverse: 5’ - TCTCAATAAAGGATAAAGCTCTCAATACT |
| acdD Full | Forward: 5’ - TAATAAGACTCACTATAAGGATAAAGCTCTCAATACT  
             Reverse: 5’ - TCTCAATAAAGGATAAAGCTCTCAATACT |
| ccdA Full | Forward: 5’ - TAATAAGACTCACTATAAGGATAAAGCTCTCAATACT  
             Reverse: 5’ - TCTCAATAAAGGATAAAGCTCTCAATACT |
| phdD Full | Forward: 5’ - TAATAAGACTCACTATAAGGATAAAGCTCTCAATACT  
             Reverse: 5’ - TCTCAATAAAGGATAAAGCTCTCAATACT |
| rbll. Full | Forward: 5’ - TAATAAGACTCACTATAAGGATAAAGCTCTCAATACT  
             Reverse: 5’ - TCTCAATAAAGGATAAAGCTCTCAATACT |
| rpaA Full | Forward: 5’ - TAATAAGACTCACTATAAGGATAAAGCTCTCAATACT  
             Reverse: 5’ - TCTCAATAAAGGATAAAGCTCTCAATACT |
| petA Full | Forward: 5’ - TAATAAGACTCACTATAAGGATAAAGCTCTCAATACT  
             Reverse: 5’ - TCTCAATAAAGGATAAAGCTCTCAATACT |

(a) - "Full" transcripts containing about 100 nts from the 5’ and 3’ UTR sequences (reviewed in Marin-Navarro et al., 2007).

Note: The consensus T7 RNA polymerase site is underlined.