Functional and molecular characterization of the frataxin homolog from *Arabidopsis thaliana*☆,☆☆

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Abstract Frataxin is a highly conserved protein from bacteria to mammals that has been proposed to participate in iron–sulfur cluster assembly and mitochondrial iron homeostasis. In higher organisms, the frataxin gene is nuclear-encoded and the protein is required for maintenance of normal mitochondrial iron levels and respiration. We describe here *AtFH*, a plant gene with significant homology to other members of the frataxin family. Plant frataxin has five segments of beta regions and two alpha helices, which are characteristics of human frataxin, as well as a potential N-terminal targeting peptide for the mitochondrial localization. Transcription analysis showed that *AtFH* is ubiquitously expressed with high levels in flowers. Complementation of a *Saccharomyces cerevisiae* mutant (*Δ*yfh) lacking the frataxin gene proved that AtFH is a functional protein, because it restored normal rates of respiration, growth and sensitivity to H_2O_2 of the null mutant. Our results support the involvement of AtFH in mitochondrial respiration and survival during oxidative stress in plants. This is the first report of a functional frataxin gene in plants.

Keywords: Arabidopsis; Frataxin; Mitochondria

1. Introduction

Elucidation of the regulatory genes controlling mitochondrial biogenesis in eukaryotic organisms, particularly in higher plants, is far from complete [1,2]. One example of this situation is illustrated by the nuclear-encoded protein frataxin. The deficiency of this protein was initially described as the phenotype in the Friedreich's ataxia (FA), an autosomal recessive disease in humans [3]. Frataxin is highly conserved from bacteria to mammals and plants, without major structural changes; this conservation suggests that frataxin could play a similar role in all these organisms, but its precise function remains unclear. Recently, it has been predicted that this protein assists in iron–sulfur cluster assembly [4,5]. Other reports suggest the involvement of frataxin in energy conversion and oxidative phosphorylation [6]. Frataxin deficiency results in impaired mitochondrial iron efflux [7], loss of ATP synthesis and deficiency in antioxidant defenses [8]. This situation might lead to iron accumulation within mitochondria inducing increased oxidative damage [9]. More recently, a ferroxidase activity was reported for yeast frataxin homolog; this evidence supports a direct role for this protein in iron metabolism [9]. Thus, several functions have been described for frataxin in mitochondria, but its specific role(s) remains elusive.

Most knowledge of frataxin is based on studies performed in mammals and yeast [6,10]. No information about frataxin homolog from photosynthetic organisms is available, with the exception of related sequences found in genome databases. We present here the first report characterizing a frataxin gene from the higher plant *Arabidopsis thaliana*.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana (var. Columbia Col-0) grown in a greenhouse were used in these experiments.

2.2. Strains

Saccharomyces cerevisiae wild-type and the strain Δ*yfh* null mutant (deficient in the expression of YFH, kindly provided by Dr. Jerry Kaplan, Utah State University) were used for the complementation analysis.

2.3. RNA extraction and cDNA synthesis

Total RNA from *A. thaliana* flowers was extracted using Trizol reagent (Invitrogen). cDNA was synthesized using either random hexamers or specific primers. The conditions used were those described in the Access RT-PCR system first strand protocol (Promega). RT-PCR quantification was performed by analyzing the reaction products after 8, 12, 16, 20, 24 and 35 cycles to obtain data during the exponential phase of the PCR reaction. PCR conditions were as follows: 94 °C for 1 min; 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 2 min and finally, 5 min at 72 °C. The PCR products were electrophoresed on agarose gels and transferred onto Hybond N+ membranes (AmershamPharmacia). Probe labeling and membrane hybridization were performed according to the ECL Direct Nucleic Acid Labeling and Detection System protocol (Amersham Pharmacia).

2.4. Molecular cloning of AtFH

Full-length (564 bp) cDNA corresponding to *AtFH* (At4g03240) was obtained by RT-PCR using *atfhup*: 5′-AAACATATGGCTA-

☆ Accession number AY649366 (*AtFH*).
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CAGCTTCAAGG-3′ (*Nde*I site underlined) and *atfhdo* primers. The resulting PCR products were subcloned into the pGEMT Easy vector (Promega) according to the manufacturer's protocol; XL1Blue *E. coli* cells were transformed and 3 out of 15 white colonies were selected. DNA sequences were verified using the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems). The *A. thaliana* frataxin cDNA sequence was submitted to Genbank (Accession number AY649366).

2.5. Similarity searches and evolutionary analysis

Using AY649366 as a search query, a set of related sequences were aligned using the program CLUSTALW under default parameters settings [11]. This alignment was used to obtain a maximum parsimony topology with the program PROTPARS of PHYLIP [12]. To obtain branching point statistical support, bootstrapping (100 re-samplings) was performed with the programs SEQBOOT and CONSENSE (see supplementary data).

2.6. Functional complementation of yeast null mutants with AtFH

A chimeric construct contained the *S. cerevisiae* cytochrome oxidase subunit IV (COXIV) transit peptide fused to the 5′end of the frataxin conserved domain. The COXIV transit peptide sequence was obtained by PCR with primers *coxup* 5′-CAGGGATCCATGCTTTCAC-TACGTCAA-3′ (containing a *Bam*HI site, underlined) and *coxdo* 5′-AAACATATGGGTACCCTCTTTAGCACC-3′ (containing a *Nde*I site, underlined). The *AtFH* ORF was amplified from a cDNA clone using the primers *atfh2*, (5′-GGGCATATGGAGGAAGAGTTT-CACAAA-3′, containing a *Nde*I restriction site, underlined) and *atfhdo* (5′-AAACTCGAGTTATGAGAGTTGG ATTGGTTC-3′, containing a *Xho*I site, underlined). After endonuclease digestion, the respective PCR products were joined with T4 ligase (Promega) and the chimeric DNA (*COXIV-AtFH*) was recovered by PCR using *coxup* and *atfhdo* primers. The recovered product was cloned into *Bam*HI and *Xho*I sites of pYES3CT vector (Invitrogen). This construct was used to transform wild-type and Δ*yfh S. cerevisiae* cells. Transformed yeast was grown in YPD medium or drop out medium without tryptophan in the presence or absence of galactose. Cellular respiration was quantified using a Clark-type oxygen electrode at 28 °C. To determine the sensitivity of the cells to growth on H_2O_2, aliquots of the yeast culture were inoculated in medium containing 0.5 mM of H_2O_2. After 30 min of incubation at 30 °C with shaking, the cells were spotted on YPD medium or drop out medium without tryptophan and allowed to grow for 48 h at 30 °C.

3. Results

3.1. AtFH is a 187 aa peptide and possesses a mitochondrial targeting sequence

Using yeast frataxin (*YFH*) as the starting sequence, we searched for homologous genes in the *Arabidopsis* Genomic Database at the National Center for Bio-technology Institute (NCBI). A predicted frataxin homolog, At4g03240, located on chromosome 4 of the *Arabidopsis* genome, predicts a protein of 143 amino acid residues, which is shorter than the mammal and yeast proteins. Attempts to clone its cDNA by RT-PCR were unsuccessful using primers designed based on data bank sequences. When oligonucleotides spanning a downstream ATG with an eukaryotic consensus of translation initiation were used, amplification was possible; this result indicates that the gene prediction was inaccurate.

The isolated cDNA (564 bp) encodes a 187 amino acid protein. Using the Protean module from DNAStar (Lasergene), we confirmed the existence of 5 segments with Beta pleated sheet characteristics and two alpha helices corresponding to a frataxin-like domain. Predictive analysis using the PSORT program or MITOPROT on the AtFH amino acidic sequence identified a likely mitochondrial localization (scores 0.751 and 0.9984, respectively) domain of 31 amino acids. Mitochondrial localization has been demonstrated for both the human and yeast frataxins [13–15]. Interestingly, AtFH possesses an Arg at position 177 as found in other plant and the yeast predicted genes as well as in the bacterial homolog CyaY [16]. In the human frataxin, a His residue involved in iron binding is found at this position [17].

The protein encoded by the *AtFH* cDNA shares important homology with other members of the frataxin family, especially at the central and C-terminal regions of the protein. High conservation was found when compared to animal frataxin: *Mus musculus* (65% similarity); *Homo sapiens* (65%); and *Drosophila melanogaster* (61%); *Caenorhabditis elegans* (57%). Conservations extend to fungi: *Schizosaccharomyces pombe* (61%), *S. cerevisiae* (66%). Interestingly, while the eubacteria *E. coli* frataxin show only 55% similarity with AtFH, the gene from *Rickettsia prowazekii* has 65% similarity, as high as mammals. This observation is in good agreement with the endosymbiotic hypothesis of a common ancestor at the origin of contemporary α-proteobacteria and mitochondria [18] (see supplementary data).

3.2. Induction in the expression of AtFH in wt and u-atp9 lines

Frataxin is differentially expressed with higher levels in tissues heavily dependent on oxidative respiration [19]. To evaluate the possible physiological role of AtFH, it is important to know the expression pattern in different *Arabidopsis* organs. Once the presence of one copy of *AtFH* was confirmed by DNA Blot (data not shown) and no second copy is evident in the available genomic sequences, we performed semi-quantitative RT-PCR experiments on RNA from leaves, roots and flowers. RT-PCR of 18S rRNA was used in parallel as an internal standard. As shown in Fig. 1A, *AtFH* was expressed in all three sources. The steady-state levels of *AtFH* mRNA were 3–4-fold higher in flowers, a high energy demanding tissue in plants, compared to leaves or roots. This result is in good agreement with the postulated function of frataxin in mitochondrial energy metabolism. In fact, the importance of

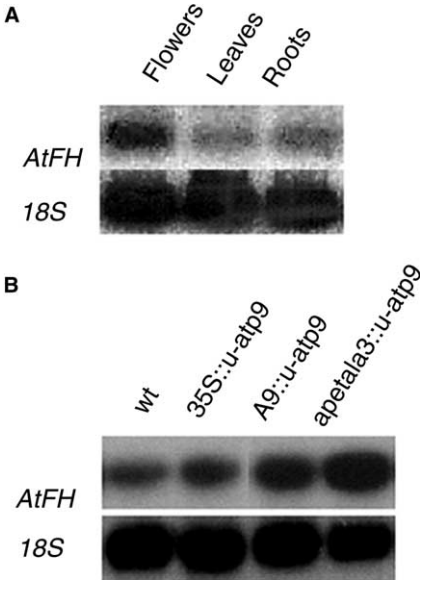

Fig. 1. (A) Expression of *AtFH* determined by RT-PCR. Total RNA was extracted from flowers, leaves or roots from 30-day-old *Arabidopsis thaliana* plants. (B) RT-PCR analysis of *AtFH* in 30-day-old flowers of wt and *u-atp9* expressing lines under the control of three different promoters, *CaMV35S::u-atp9*, *apetala3::u-atp9 and A9::u-atp9*. 18S rRNA was used as internal marker.

mitochondrial function during flower development is well known, in particular during sporogenesis and pollen maturation [20].

Recently, we reported the specific induction of three nuclear-encoded mitochondrial genes: *PSST*, *TYKY* and *NADHBP* in *Arabidopsis* "*u-atp9*" lines (which exhibit a mitochondrial dysfunction) [21]. These proteins are constituents of the electron transport complex I (nCI) in plants. The "*u-atp9*" lines constructed by transformation of *A. thaliana* (var Columbia) with recombinant vectors carrying the *u-atp9* gene under the control of the *35S CaMV*, *apetala3*, or *A9* promoters were used to analyze *AtFH* mRNA expression levels in flowers. We found that *AtFH* is strongly transcribed in flowers from "*u-atp9*" plants, especially in plants in which the transgene is expressed under the control of A9 (2.8-fold) and *apetala3* (4.1-fold) promoters (Fig. 1B). These results are in parallel to previous results found for nCI proteins [21].

3.3. Δ*yfh* cells expressing AtFH shows normal rates of growth, respiration and sensitivity to H_2O_2

Previous studies have suggested a decrease in mitochondrial respiration following inactivation of the frataxin gene in yeast [19,22]. It was also determined that in null mutant Δ*yhf* cells, there is an increase in mitochondrial iron content, making this strain hypersensitive to oxidative stress [10].

To study the function of AtFH, we evaluated its ability to restore normal phenotypes to the Δ*yfh* yeast mutant. Cells containing the chimeric construct *COXIV-AtFH* were prepared as described in materials and methods. No significant growth differences were found when comparing complemented Δ*yfh* cells with wild-type yeast on standard media (data not shown). Δ*yfh* cells expressing *AtFH* had about 2-fold greater O_2 uptake compared to the null mutant (1.8 ± 0.2 and 0.82 ± 0.1 nmol O_2/min/10^6 cells, respectively; Fig. 2). Our results clearly show that mutant cells overexpressing a plant frataxin exhibit normal rates of respiration, suggesting that an increased electron transport activity occurs in Δ*yfh* complemented cells.

To ascertain the role of plant frataxin, we evaluated the sensitivity to oxidative stress of Δ*yfh* yeast cells complemented with *COXIV-AtFH*. This strategy is based on the fact that yeast lacking YFH are more sensitive to oxidants [8,10,23]. Wild-type, Δ*yfh* and Δ*yfhAtFH* cells were cultured on medium containing 0.5 mM H_2O_2, then transferred to complete YPD medium. Lower rates of growth and high sensitivity to hydrogen peroxide were observed for the null mutant compared to wt. By contrast, Δ*yfh* complemented cells had a normal growth. In the presence of H_2O_2, complemented null mutants were more resistant to induced oxidative stress than the mutant (Fig. 3). Furthermore, preliminary results obtained by infiltration of leaves with 500 μM H_2O_2 showed that *AtFH* is induced 4–5-fold (4 and 8 h after treatment) compared to control leaves infiltrated with water (data not shown). Considered together, these results support a protective role for AtFH against the external oxidant H_2O_2.

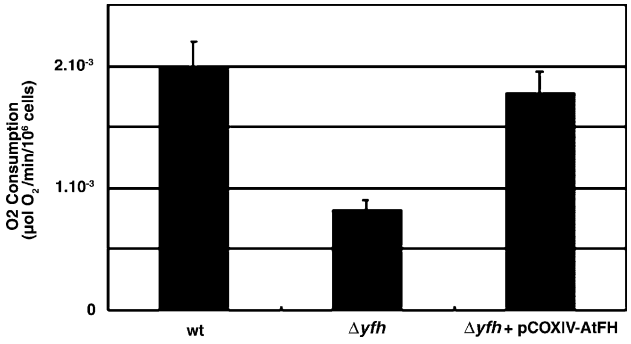

Fig. 2. Respiratory rate of wild-type *S. cerevisiae* cells (wt), frataxin null mutant (Δ*yfh*), and the frataxin null mutant transformed with a plasmid containing the AtFH coding region fused to a yeast COXIV transit peptide (Δ*yfh* + pCOXIV-AtFH).

4. Discussion

Frataxin is necessary for normal mitochondrial function in yeast and human cells [6,10]. Yeast strains lacking the *Yfh1p* gene have defective mitochondrial respiration, exhibit loss of mtDNA, and accumulate higher levels of mitochondrial iron [10]. This protein has been conserved through evolution from bacteria to mammals. AtFH, the first plant protein demonstrated to have frataxin activity, shares important homology with other members of the frataxin family. This suggests that different frataxin homologs play common biological functions, but its precise role remains unclear. The 3D structures of the human and the bacterial gene products (HFH and CYAY) contain a novel protein fold. Indeed, these folding properties seem to be characteristic of frataxin and the related homologs because no other proteins present in databases share such

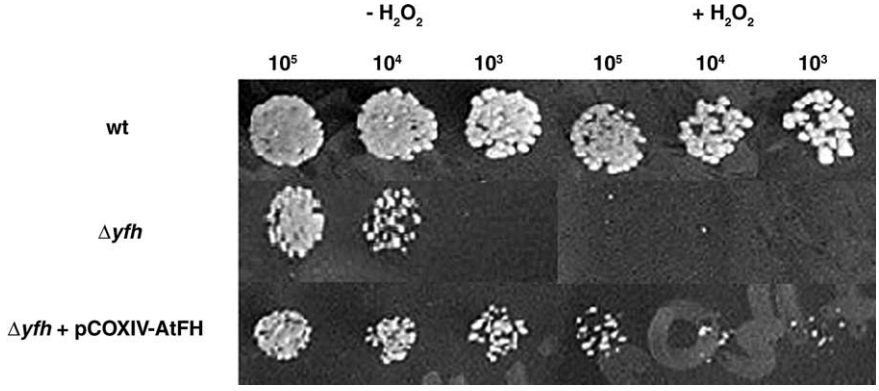

Fig. 3. Sensitivity to oxidative stress of Δ*yfh* and wild-type *S. cerevisiae*. After incubation for 30 min in culture medium containing H_2O_2, serial dilutions of 10^5–10^3 cells/5 μl were spotted on YPD medium and grown for 60 h at 30 °C.

motifs [16,24]. Thus, it becomes difficult to postulate possible functions only through structural comparisons.

In the present work, we identified the first frataxin protein in a photosynthetic organism. The putative frataxin homolog from *A. thaliana* has an ORF of 564 bp encoding 187 residues. Based on in silico analysis, we postulate that this protein is targeted to mitochondria. It is interesting to note that AtFH has an equally high similarity to human and *R. prowazekii* frataxin suggesting that the contemporary nuclear-encoded *Arabidopsis* frataxin gene originated from an ancestral α-proteobacterial genes, in accord with the endosymbiotic theory [18]. In fact, the main difference between frataxin homologs is evident at the N-terminal portion of the protein, which contains the mitochondrial target signal. As expected, eubacterial frataxins lack such N-terminal extensions.

Significant sequence similarity with human frataxin was documented by sequence comparisons (see supplementary data). The strongest evidence supporting the hypothesis that the *AtFH* gene is a frataxin was obtained by complementation experiments in yeast. AtFH restores the growth and respiratory capacity of Δ*yfh* cells, a frataxin null-mutant yeast.

In mammals, frataxin is expressed in tissues with high metabolic activity, such as brown fat, heart, liver and neurons [6,19]. We observed a higher transcript level of this gene in flowers, a high energy demanding tissues in plants [20]. Moreover, *AtFH* expression was further increased in flowers from *A. thaliana* lines showing a mitochondrial dysfunction induced by the expression of the unedited version of ATP synthase subunit 9. Previously, we demonstrated that the mitochondrial flaw in these plants results in higher expression of at least three nuclear-encoded mitochondrial complex I genes, *TYKY*, *PSST* and *NADHBP*. Previously, it has also been reported that the normal induction of these genes is 6–10-fold times higher in flowers than in other plant organs [25–27]. In flowers, particularly in tapetal cells of anthers, there is an intense mitochondrial activity during pollen development [20]. This constitutes one of the major energy consuming processes in plants. The induction of *AtFH* in flowers is in agreement with its proposed role in mitochondrial respiration. One possibility is that this is a compensatory nuclear response, by which affected mitochondria increased mitochondriogenesis. The compensatory response was unable to rescue the male sterile phenotype shown by *u-atp9* lines [21]. Thus, the induction of *AtFH* expression in these lines could also be interpreted as a nuclear compensation in response to a decrease in mitochondrial respiration.

Considering that the oxidant sensitivity characteristic of yeast null mutant Δ*yfh* was corrected by the expression of plant frataxin, it is possible that the induction of *AtFH* in respiratory-deficient plants reflects a response of the cell to increasing reactive oxygen species (ROS) generated by impaired mitochondrial respiration. Preliminary results using two Salk mutants (SALK_021263 and SALK_094203) with defects in frataxin expression showed a delay in growth and a mild sterile phenotype, consistent with the proposed role of AtFH. Taken together, these results suggest that AtFH is a plant frataxin involved in normal mitochondrial respiration and in oxidative stress in plant cells.

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