Characterization of a ferritin mRNA from *Arabidopsis thaliana* accumulated in response to iron through an oxidative pathway independent of abscisic acid

Frédéric GAYMARD, Jossia BOUCHEREZ and Jean-François BRIAT*

Biochimie et Physiologie Moléculaire des Plantes, Centre National de la Recherche Scientifique (Unité de Recherche 2133), Institut National de la Recherche Agronomique et École Nationale Supérieure d'Agronomie, Place Viala, F-34060 Montpellier, cedex 1, France

A ferritin cDNA, *AtFer1*, from seedlings of *Arabidopsis thaliana* has been characterized. The deduced amino acid sequence of the AtFer1 protein indicates that *A. thaliana* ferritin shares the same characteristics as the plant ferritin already characterized from the *Leguminosae* and *Graminacea* families: (i) it contains an additional sequence in its N-terminal part composed of two domains: a transit peptide responsible for plastid targeting and an extension peptide; (ii) amino acids that form the ferroxidase centre of H-type animal ferritin, as well as Glu residues characteristic of L-type animal ferritin, are conserved in AtFer1; (iii) the C-terminal part of the *A. thaliana* ferritin subunit defining the E-helix is divergent from its animal counterpart, and confirms that 4-fold-symmetry axis channels are hydrophilic in plant ferritin. Southern blot experiments indicate that *AtFer1* is likely to be encoded by a unique gene in the *A. thaliana* genome, although a search in the NCBI dbEST database indicates that other ferritin genes, divergent from *AtFer1*, may exist. Iron loading of *A. thaliana* plantlets increased ferritin mRNA and protein abundance. In contrast to maize, the transcript abundance of a gene responding to abscisic acid (*RAB18*) did not increase in response to iron loading treatment, and *A. thaliana* ferritin mRNA abundance is not accumulated in response to a treatment with exogenous abscisic acid, at least in the culture system used in this study. In addition, iron-induced increases in ferritin mRNA abundance were the same as wild-type plants in *abi1* and *abi*2 mutants of *A. thaliana*, both affected in the abscisic acid response in vegetative tissues. Increased *AtFer1* transcript abundance in response to iron is inhibited by the antioxidant *N*-acetylcysteine. These results indicate that an oxidative pathway, independent of abscisic acid, could be responsible for the iron induction of ferritin synthesis in *A. thaliana*.

INTRODUCTION

Ferritins are high-molecular-mass multimeric proteins (24-mers) which are able to accommodate up to 4500 iron atoms in their central cavity [1–3]. Redox processes, responsible for the regulation of iron uptake and release by these proteins, fulfil metabolic iron requirements, avoiding insolubility and toxicity of this element in the presence of oxygen [1]. Despite specific structural differences, plant and animal ferritins arise from a common ancestor [4] and have important similarities in their primary sequence as well as in their secondary and tertiary structures [3,5]. Major differences, however, do exist between plant and animal ferritins. These concern subcellular localization and regulation of their synthesis in response to iron. Animal ferritins are cytoplasmic proteins, whereas plant ferritins are found within plastids [6,7]; animal and plant ferritins are both encoded by nuclear gene families which diverge in their intron/exon organization [8,9]. Although a minor transcriptional control has been reported to regulate expression of animal genes encoding the L-type ferritin subunit, the major level of regulation of animal ferritin synthesis occurs at the translational level [10–13]. Translational repression occurs at low intracellular iron concentrations and is mediated by the binding of iron regulatory proteins (IRPs) to iron regulatory elements (IREs) found in the 5′ untranslated region (UTR) of ferritin mRNA; iron loading of animal cells results in IRPs release from IREs, allowing recruitment of ferritin mRNA on to polysomes and translation. In contrast, plant ferritin mRNA accumulates in response to iron overload [7,14] and, at least in the case of cultured soybean cells, this regulation has been shown to take place at the transcriptional level [7]. No molecular mechanisms responsible for this control have been reported so far. However, in maize, two independent transduction pathways involved in the iron induction of ferritin synthesis have been demonstrated. One of these pathways involves the plant hormone abscisic acid (ABA) as a cellular relay [15]. ABA is a 15-atom sesquiterpene synthesized by cleavage of epoxycarotenoids (violaxanthin, zeaxanthin, etc.) to xanthoxin which is then converted into ABA-aldehyde by xanthoxin oxidase; ABA-aldehyde then yields ABA through the action of an aldehyde oxidase [16,17]. ABA is ubiquitous among higher plants where it is involved in the control of many aspects of plant growth and development. In seeds, ABA regulates the accumulation of protein and lipid reserves, the induction of dormancy, and the acquisition of resistance to desiccation. In vegetative tissues, ABA regulates many physiological and molecular responses of plants to environmental stresses such as drought or high salinity [18,19]. Plant genes whose expression is regulated by ABA are known as RAB genes (responsive to abscisic acid) [20]; among these genes, the RAB18 gene is known to participate in the cold acclimation process of *Arabidopsis thaliana* [21]. The second pathway involved in maize ferritin synthesis in response to iron implicates reactive oxygen inter-

Abbreviations used: ABA, abscisic acid; RAB genes, plant genes responsive to abscisic acid; NAC, *N*-acetylcysteine; UTR, untranslated region; IRPs, iron regulatory proteins; IRE, iron regulatory element; EST, expressed sequence tags.

* To whom correspondence should be addressed.

The nucleotide sequence of AtFer1 has been deposited in the EMBL Data Library under the accession number X94248.

mediates [22]. Whether these pathways operate in dicotyledonous plants is unknown. In order to address this question we have now characterized an *A. thaliana* ferritin cDNA (*AtFer1*) isolated during the course of an EST (expressed sequence tags) program [23]. *A. thaliana* is a model dicotyledonous plant for molecular genetic studies [24]. It is relatively easy to produce numerous chemical and tagged mutants, to map them on this small genome, and to clone the corresponding genes. A huge international effort to establish the genetic map, to characterize randomly obtained EST and to sequence the genome of *A. thaliana*, has already brought a wealth of information concerning various pathways involved in plant metabolism, in plant stress responses and in plant development [24]. Using liquid-cultured *A. thaliana* seedlings, we demonstrate that, in such a system, the *AtFer1* transcript is accumulated in response to iron through an oxidative pathway, which does not involve ABA.

MATERIALS AND METHODS

Plant culture

Columbia ecotype was used as wild type, as well as *abi1* and *abi2* mutants [25–28], the seeds of which was kindly provided by Dr. Parcy (ISV, Gif sur Yvette, France). Seeds were surface-sterilized by soaking for 15 min in 1.5 % calcium hypochlorite, 0.05 % Tween. Seedlings were grown in 250 ml Erlenmeyer flasks containing 100 ml of MS medium (Sigma), pH 5.7, 2 % glucose and 0.5 g/l of Mes. After 1 week of culture at 25 °C under continuous light ($100\ \mu E \cdot s^{-1} \cdot m^{-2}$) on a rotating table (60 rev./min), medium was discarded and plantlets were extensively washed with 0.2 mM $CaSO_4$ prior to adding 100 ml of MS without iron. Plants were grown for a further week under these iron-starved conditions. After this period, plants were treated for various times with iron (500 μM $FeSO_4$/1 mM trisodium citrate), or with 200 μM ABA (Sigma) as previously described [15]. *N*-Acetylcysteine (NAC; Sigma) treatment, and co-treatment with iron citrate, were also performed as described [22]. After washing with 10 mM KCl/1 mM EDTA, pH 8.0, samples were collected and frozen in liquid nitrogen prior to storage at −70 °C.

Cloning and sequencing

The *AtFer1* cDNA had been obtained during the course of the French *A. thaliana* EST program (accession numbers Z18109 and Z30743). It was kindly provided by Dr. Desprez (Laboratoire de Biologie Cellulaire, INRA, Versailles, France), as a cDNA cloned in the *Not*I site of the pRD-1 vector [23]. Classical molecular biology methods were used according to Ausubel et al. [29]. The DNA insert of about 1 kbp was subcloned into the *Not*I site of the pBS-SK vector. A restriction map of this DNA fragment indicated a unique *Eco*RI site in its middle. Both *Not*I–*Eco*RI fragments were subcloned in the pBS-SK vector. A series of exonuclease III nested deletions were generated according to Henikoff [30]. Both strands of the cDNA were sequenced by the dideoxy method [31] using the T7 sequencing kit (Pharmacia). Sequence analysis was carried out by using the DNA Strider II software and by connecting to the BLAST Network Service of NCBI.

Genomic DNA analysis

Genomic DNA from *A. thaliana* seedlings (var. Columbia) was prepared according to the method of Dellaporta et al. [32]. Samples (2 μg) of DNA were digested by various enzymes for 4 h at 37 °C, and electrophoresed on to a 0.4 M Tris-acetate/0.002 M EDTA (TAE)/0.7 % agarose gel. After depurination by soaking the gel in 0.25 M HCl, denaturation in 0.5 M NaOH/1.5 M NaCl, and neutralization by 0.5 M Tris, pH 7.2/1.5 M NaCl/1 mM EDTA, DNA was transferred to nylon filters (Hybond N, Amersham). Hybridization with the ^{32}P-labelled probe A (see Figure 3), and washes were as described [8]. Autoradiography was performed for 3 days at −70 °C to Royal X-Omat film (Kodak) with an intensifying screen.

RNA preparation and analysis

RNA preparations and analysis by Northern blots were as described [8]. Two DNA probes were used: the *Not*I–*Eco*RI fragment including the 5′ UTR and the first 510 bp of the coding sequence, and a 3′ UTR probe (starting 4 bp downstream of the stop codon) obtained as one of the nested deletions generated by exonuclease III (see Figure 3). Filters were exposed for a few hours at −70 °C to Royal X-Omat film (Kodak) with an intensifying screen.

Protein preparation and analysis

Total protein extracts were prepared from 1 g of each sample as described [14]. Protein concentrations were measured according to Schaffner and Weissmann [33] using BSA as standard. Proteins were subjected to electrophoresis on a 15 % polyacrylamide gel/0.1 % SDS according to the method of Laemmli [34]. Ferritin protein used as positive control was a purified *Escherichia coli* recombinant pea seed ferritin [35]. After electroblotting the protein on to a nitrocellulose filter (Sartorius), immunodetection of ferritin was performed using a rabbit polyclonal antiserum raised against purified pea seed ferritin [36] and the ICN Aurora Western blotting kit.

Iron measurement

Samples were mineralized at 250 °C in the presence of concentrated hydrochloric, nitric and perchloric acids. Total iron concentration was estimated spectrophotometrically at 535 nm using bathophenanthroline, after reduction of the samples with thioglycollic acid, as described [22].

RESULTS

Characterization of the ferritin cDNA *AtFer1* from *A. thaliana*

The *AtFer1* cDNA was obtained during the course of the French *A. thaliana* EST program. It had been randomly picked from a cDNA library of 5-day-old etiolated seedlings constructed in the pRD-1 vector [23]. Analysis of the 5′ partial sequence, 396 nucleotides, identified homology to a soybean ferritin precursor [7]. In order to deduce the amino acid sequence of the *A. thaliana* ferritin we determined the complete sequence of the *AtFer1* cDNA (Figure 1).

The sequence of *AtFer1* is 989 nucleotides long and contains an open reading frame of 246 amino acids which is preceded by a 5′ UTR of 38 nucleotides. A 3′ UTR of 183 nucleotides is found downstream of a UAG stop codon found at position 804; no polyA$^+$ tail was found. The open reading frame starts in a favourable context for translation initiation [37]. A sequence homologous to the plant ferritin extension peptide sequence starts at amino acid 51 [4,5], and the sequence (Figure 1) has homology to the common part of plant and animal ferritin amino acid sequences from amino acid 84 onwards (Figure 2). Indeed, the *A. thaliana* ferritin mature subunit encoded by *AtFer1* shares between 66 % (MaIR2) and 73 % (SoIC) identity

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1    aggcggctcaacactatcctcttctacattttccaacg ATG GCC TCA AAC GCA
1                                           M   A   S   N   A

54   CTC TCG TCT TTC ACC GCC GCT AAT CCC GCT CTG TCT CCT AAG CCA
6     L   S   S   F   T   A   A   N   P   A   L   S   P   K   P

99   CTA CTC CCT CAC GGC TCT GCT TCT CCG TCG GTT TCT CTC GGC TTC
21    L   L   P   H   G   S   A   S   P   S   V   S   L   G   F

144  TCC AGG AAA GTT GGC GGC GGC AGA GCA GTG GTC GTT GCA GCG GCT
36    S   R   K   V   G   G   G   R   A   V   V   V   A   A   A

189  ACG GTG GAC ACA AAC AAC ATG CCG ATG ACC GGA GTC GTG TTC CAG
51    T   V   D   T   N   N   M   P   M   T   G   V   V   F   Q

234  CCT TTC GAA GAG GTG AAG AAA GCC GAT CTG GCC ATT CCA ATC ACA
66    P   F   E   E   V   K   K   A   D   L   A   I   P   I   T

279  TCT CAT GCC TCT CTC GCT CGC CAG AGG TTT GCC GAC GCT AGC GAG
81    S   H   A   S   L   A   R   Q   R   F   A   D   A   S   E

324  GCA GTC ATT AAT GAG CAA ATC AAT GTG GAA TAC AAC GTC TCC TAT
96    A   V   I   N   E   Q   I   N   V   E   Y   N   V   S   Y

369  GTG TAC CAT TCA ATG TAC GCA TAC TTT GAC AGA GAC AAC GTT GCT
111   V   Y   H   S   M   Y   A   Y   F   D   R   D   N   V   A

414  ATG AAG GGA CTA GCC AAA TTT TTC AAG GAA TCA AGT GAG GAA GAG
126   M   K   G   L   A   K   F   F   K   E   S   S   E   E   E

459  AGA GGG CAT GCT GAG AAG TTT ATG GAG TAC CAG AAC CAA AGA GGA
141   R   G   H   A   E   K   F   M   E   Y   Q   N   Q   R   G

504  GGA AGA GTG AAA CTC CAC CCT ATC GTC TCA CCT ATC TCA GAA TTC
156   G   R   V   K   L   H   P   I   V   S   P   I   S   E   F

549  GAA CAT GCT GAA AAA GGA GAT GCT TTA TAT GCA ATG GAG TTG GCT
171   E   H   A   E   K   G   D   A   L   Y   A   M   E   L   A

594  CTG TCT CTA GAG AAA CTC ACT AAT GAG AAG CTT CTA AAC GTT CAC
186   L   S   L   E   K   L   T   N   E   K   L   L   N   V   H

639  AAA GTG GCC TCA GAG AAC AAT GAT CCC CAG TTA GCT GAT TTC GTT
201   K   V   A   S   E   N   N   D   P   Q   L   A   D   F   V

684  GAG AGT GAA TTT CTG GGA GAG CAG ATT GAA GCA ATC AAG AAG ATC
216   E   S   E   F   L   G   E   Q   I   E   A   I   K   K   I

729  TCA GAC TAC ATC ACC CAG CTA AGG ATG ATC GGC AAA GGC CAC GGA
231   S   D   Y   I   T   Q   L   R   M   I   G   K   G   H   G

774  GTT TGG CAT TTC GAC CAG ATG CTT CTG AAC TAG acttggacctctata
246   V   W   H   F   D   Q   M   L   L   N   Z

822  agttcactttcagatgaccaatgttggggttaaaacagaagaacccgaagattctgtaa
881  aagctttagattgaacattatggactaataagattgtagtagtaagctttgtgttcaag
940  atggtttttgtaatatacttcgtaagcttaatcaatgaagtttttgtttta
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Figure 1 Nucleotide and deduced amino acid sequences of the *AtFer1* ferritin cDNA from *A. thaliana*

Lower-case letters indicate the 5′ and 3′ UTRs and upper-case letters the open reading frame. The putative transit peptide cleavage site is underlined. The nucleotide sequence of *AtFer1* has been deposited in the EMBL Sequence Library under the accession number X94248.

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                        20                  40                  60
ATFER1 TVDTNNMPMTGVVFQPFEEVKKADLAIPITSHASLARQRFADASEAVINEQINVEYNVSYVYHSMYAYFD
PeSd1      TTA-L---I-E-------DY--V-SVPLV-----N---EC-S----------A------LF----
PeSd2      TTA-L---I-E-------DY--V-SVPLV-----N---EC-S----------V------MF----
SoIC-      STV-L---I-E-------SE--V-TAPQV-----NY--EC-SA---------A------LF----
Fb-S       STV-L---I-E--------E--V-TAGQV-----YY--EC-SA---------A------LF----
MaIR1  AAGKGKEVLS---------I-GELALV-QSPDK---RHK-V-DC--AL---------A--A---LF----
MaIR2  AAGKGKEVLS---------I-GELALV-QSPDR---RHK-V-DC--A----------A--A---LF----
HoS-L                                  S-QI--NYSTEV--AV-RLV-LYLRA--T-L-LGF---
HuL-H                                T-AST-QV--NYHQD---A--R---L-LYA----L--SX---
                                       13          27          34

             80                  100                 120                 140
ATFER1 RDNVAMKGLAKFPFKESSEEERGHAEKFMEYQNQRGGRVKLHPIVSPISEFEHAEKGDALYAMELALSLEK
PeSd1  -----L--F----------H-E----L-K---T-----V----KDVP-----V----------------
PeSd2  -----L--F-----------E----L-K---T-----V----KDVP-----V------H----------
SoIC-  -----L--F-----------E----L-K---T-----V----KNVP-----V----------------
Fb-S   -----L--F-R---------E----L-K---T-----V----KNVP-----V----------------
MaIR1  -----L--F--------D---E----L-----K-----R-QS--T-LT--D-P-------------A---
MaIR2  -----L--F--------D---E----L-----K-----R-QS--A-LT--D-P----------T-A---
HoS-L  --D--LE-VCH--R-LA--K-EG--RLLKM-------ALFQDLQK.P-QD-WGTTL-...--KALIV---
HuL-H  --D--L-NF--Y-LHQ-H---E----L--L-------IF-QD-KK.PDCDDWESGLN...---C--H---
                  61  65                                                107

                       160                 180                 200
ATFER1 LTNEKLLNVHKVASENNDPQLADFVESEFLGEQIEAIKKISDYITQLRMIG...KGHGVWHFDQMLLN
PeSd1  -T--------S--ER---LEMTH-I-G-Y-A--V-------E-VA---RV-...----------R--HGVHGA
PeSd2  -T--------S--ER---LEMTH-I-G-Y-A--V-------E-VA---RV-...----------R--HGVHGA
SoIC-  ----------S--DR-----M---I-----S--V-S-----E-VA---RV-...----------R--D
Fb-S   ------RS--S--DR-K--------I-----S--V-------E-VA----V-...----------S--HDGHAA
MaIR1  ------H-L-G--TRC-----T--I-----E--G---N---K-VA---RV-...-----------EEEA
MaIR2  ------HSL-G--TRC-----I--I-----E--G---N-V-K-VA---RV-...-----------Q-A-
HoS-L  SL-QA--DL-ALG-AQA--H-C--L--H--D-EVKL---MG-HL-NIQRLVGSQA-L-EYL-ERLT-KHD
HuL-H  N--QS--EL--L-TDK---H-C--I-THY-N--VK---ELG-HV-N--KM-APES-LAEYL--KHT-GDSDNES
                                  141
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Figure 2 Comparison of primary structures of some plant and animal ferritins

Plant ferritin sequences correspond to the mature subunit coding region. Upper numbers apply to amino acids from *A. thaliana* ferritin and lower italic numbers apply to the human H-chain subunit [44]. Plant ferritin sequences are from *A. thaliana* seedlings (ATFER1, this work), pea seeds (PeSd1 and PeSd2, [5] and [35]), iron-loaded cultured soybean cells (SoIC; [7]), french bean seedlings [46] and maize roots loaded with iron (MaIR1 and MaIR2; [14]). Animal sequences are from horse spleen L-ferritin (HoS-L; [47]) and human liver H-ferritin (HuL-H; [48]). Dashes indicate identical amino acids and points are for gaps.

with other plant ferritins (Figure 2). Furthermore, AtFer1 ferritin protein has 36 % identity with the human L-ferritin subunit and 48 % identity with the human H-ferritin subunit (these values were calculated from comparison of the deduced mature *A. thaliana* amino acid sequence). These sequence data verified that *AtFer1* encodes a ferritin protein. In addition, the conserved amino acids indicated in Figure 2 reveal that the structural features of eukaryotic ferritins (ferroxidase activity and nucleation of the mineral core on the cavity surface of apoferritin [2]) are conserved in *A. thaliana* ferritin (see Discussion section). The in-frame peptide of 50 amino acids found upstream of this mature ferritin subunit sequence probably encodes a chloroplast-type transit peptide, since plant ferritins are known to be nuclear-encoded proteins, synthesized as precursors which are transported to the plastids [4,7,38]. In fact, this 50-amino-acid peptide has structural characteristics of chloroplastic transit peptides as defined by von Heijne et al. [39]. It contains 50 % hydrophobic residues and no acidic residues. It starts with Met-Ala-Ser and has no charged amino acids (neither proline nor glycine) in the first ten N-terminal residues of its sequence. The number of serine residues (nine) is superior to $0.007 + 1.4\times$ the number of arginine (one). Although the length of AtFer1 transit peptide is similar to the length of other plant ferritin transit peptides [40], it shares limited identity with them (between 6 % with that of maize and 28 % with that of French bean).

A. thaliana ferritin gene family

A BLASTN search of dbEST of NCBI (release December 9th, 1995) indicated 24 *A. thaliana* ESTs sharing nucleotide identity with the *AtFer1* nucleotide sequence (Figure 1). Among these 24 ESTs, 21 have more than 90 % identity with part of the coding sequence of *AtFer1*, and are probably equivalent to the *AtFer1* transcript, assuming sequencing errors in the ESTs; among these 21 ESTs, 3′ UTR sequence is available for six of them, and match the 3′ UTR of *AtFer1*. The three others (accession numbers T76041, Z34949 and T45382) share 73 %, 71 % and 68 % identity respectively, with the coding sequence of *AtFer1*, indicating that they may originate from transcripts different to *AtFer1*.

Southern blot analysis was carried out with *A. thaliana* genomic DNA, hydrolysed by various restriction enzymes which do not have recognition sites within the 5′ *Not*I–*Eco*RI cDNA fragment used as probe (probe A in Figure 3). In the case of *Eco*RI, *Bam*HI, *Eco*RV and *Xho*I digestions (Figure 3) only one DNA fragment hybridized ranging in size between 4 and 6 kbp, whereas two *Hin*dIII genomic DNA fragments of 2.5 and 0.7 kbp respectively hybridized to the probe, which did not contain any *Hin*dIII site.

These results indicate that the *AtFer1* gene is unique, and that an *Hin*dIII site could be present in an intron, upstream of the *Eco*RI site, situated in the middle of the coding sequence. However, the existence of other *A. thaliana* ferritin genes divergent from *AtFer1* (i.e. not recognized by the *AtFer1* cDNA probe A used in this study) is plausible, as suggested by the EST database NCBI search results.

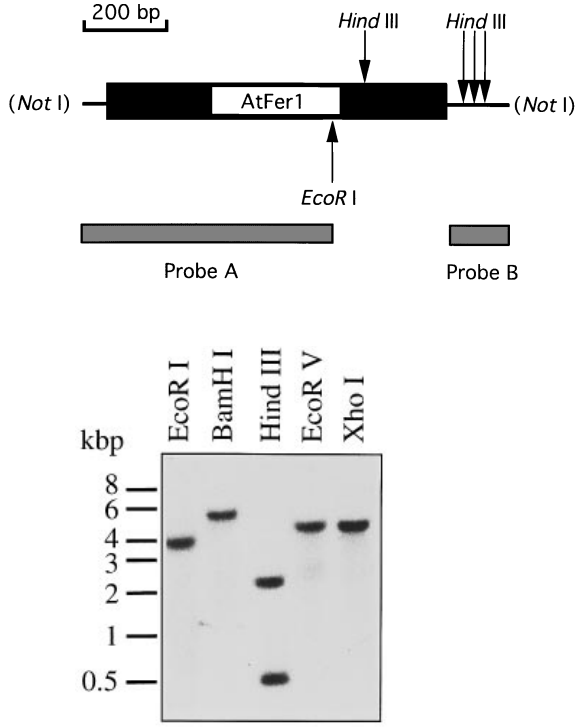

Figure 3 Southern blot analysis of the *A. thaliana* genome with an *AtFer1* cDNA probe

The upper part of the Figure is a schematic representation of the *AtFer1* cDNA; the *Not*I site is the cloning site in the pRD vector. *Eco*RI and *Hin*dIII sites are shown by vertical arrows; no *Bam*HI, *Eco*RV and *Xho*I sites are found in this cDNA. Probes A and B used in hybridization experiments are represented by grey rectangles. The lower part of the Figure shows an autoradiogram of a Southern blot of *A. thaliana* genomic DNA digested with various restriction enzymes and probed with the ^{32}P-labelled ferritin probe A. Samples (2 μg) of DNA digested with *Eco*RI, *Bam*HI, *Hin*dIII, *Eco*RV and *Xho*I were separated by electrophoresis on a 0.7% agarose gel prior to transfer on to nylon filter (Hybond N, Amersham). After hybridization with probe A overnight at 42 °C, the filter was washed in 0.1 × SSC/0.1 SDS, dried and exposed for 3 days at −70 °C to a Royal X-Omat film (Kodak) with an intensifying screen (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate).

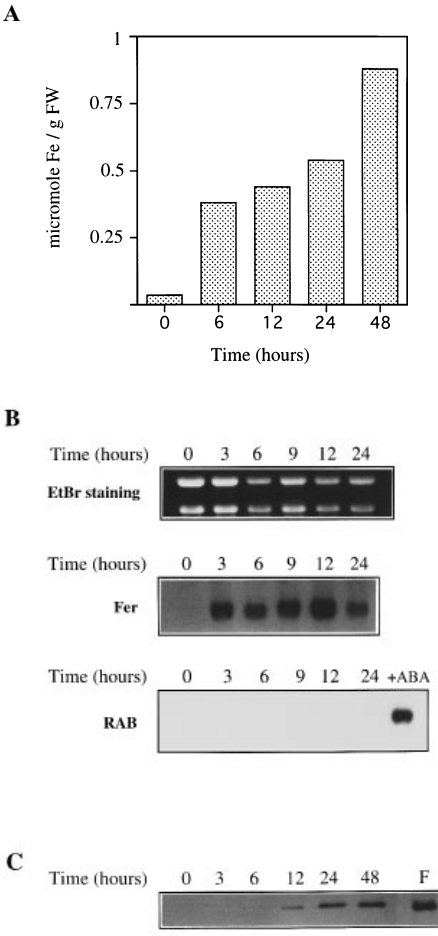

Figure 4 Iron induction of ferritin synthesis in liquid-cultured *A. thaliana* plantlets

(**A**) Measurement of iron concentration in iron-treated plantlets. Plantlets were incubated in a 500 μM $FeSO_4$/1 mM sodium citrate solution for 0, 6, 12, 24 and 48 h and iron concentration was estimated as described in the Materials and methods section; values represent the means of three different experiments and deviations at each point were below 10%. gFW, gram fresh weight. (**B**) Ferritin mRNA (Fer) and RAB18 mRNA (RAB) response to iron treatment. *A. thaliana* plantlets were grown in an iron-containing medium (see **A**) for 0, 3, 6, 9, 12 and 24 h. Aliquots (10 μg) of total RNA were fractionated by electrophoresis in an agarose/formaldehyde gel (prior to blotting on to a nylon filter (Hybond N, Amersham). After hybridization to ^{32}P-labelled probe A (Figure 3) or to ^{32}P-labelled RAB18 probe [21] for one night, the filter was washed, dried and exposed for a few hours at −70 °C on to a Royal X-Omat film (Kodak) with an intensifying screen. As a positive control for *RAB18* gene expression, the RAB probe was hybridized with an RNA sample prepared from *A. thaliana* seedlings treated for 12 h with 200 μM ABA (+ABA). (**C**) Determination of ferritin content in induced plantlets by immunodetection. Total proteins were extracted from frozen samples corresponding to plantlets untreated or treated for 6, 12, 24 and 48 h with iron. Aliquots (20 μg) were separated on SDS/PAGE. Proteins were blotted on to nitrocellulose and probed with polyclonal antibodies raised against pea seed ferritin [36]. As a control, 20 ng of purified recombinant pea seed ferritin (F) [35] was loaded.

Iron increases ferritin transcript and protein abundance in *A. thaliana*

It has previously been reported that addition of iron to culture medium of iron-starved maize plantlets and soybean cells resulted in an increase in intracellular iron concentration which correlated with induction of ferritin mRNA and protein accumulation [7,14]. In order to set up a system where the induction of ferritin synthesis in response to iron could be studied in the model plant *A. thaliana*, we first determined the conditions allowing seedlings grown in liquid medium to be loaded with iron. Addition of 500 μM iron chelated to 1 mM citrate in the culture medium of iron-starved *A. thaliana* seedlings led to a progressive increase in total plant iron content by more than 25-fold after 48 h of treatment (Figure 4A). In response to this treatment ferritin mRNA abundance increased significantly after only 3 h, with a maximum observed at 12 h followed by a decrease after 24 h (Figure 4B). Ferritin protein was also accumulated in response to the iron treatment, and was already detected 12 h after iron addition; its accumulation increased up to 48 h (Figure 4C). In conclusion, both *A. thaliana* ferritin mRNA and protein accumulate in liquid-cultured seedlings in response to high iron concentrations, with the same kinetics as reported for other plant systems [7,14].

ABA is not involved in the iron-induced increase in ferritin mRNA abundance

In maize, part of the iron response inducing ferritin mRNA accumulation is mediated through a pathway which involves the

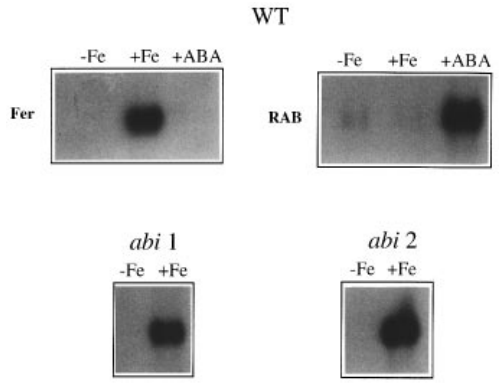

Figure 5 *A. thaliana* ferritin mRNA does not increase in response to ABA

Ferritin (Fer) and RAB18 (RAB) mRNA levels were visualized by Northern blot experiments: without iron treatment (−Fe), in response to a 12 h iron loading treatment with 500 μM iron citrate (+Fe), or in response to a 12 h treatment with 200 μM exogenous ABA (+ABA) of wild-type liquid-cultured *A. thaliana* plantlets; ferritin (Fer) mRNA abundance after a 12 h iron loading treatment of *A. thaliana abi1* and *abi2* mutants, affected the ABA response in vegetative tissues [25–28]. Ten μg of total RNA from each sample was analysed by Northern blots using ^{32}P-labelled probe A (see Figure 3).

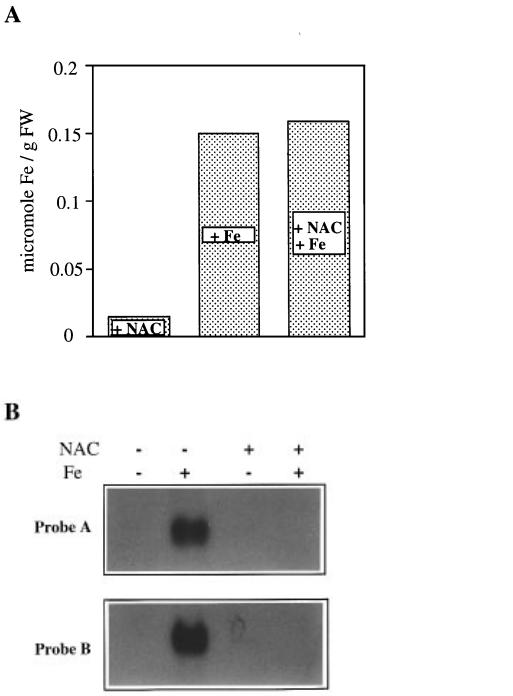

Figure 6 Inhibition of *AtFer1* transcript accumulation by an iron–antioxidant co-treatment

Liquid-cultured *A. thaliana* plantlets were treated with iron and/or an antioxidant agent as follows: 500 μM iron citrate for 3 h (+Fe); 10 mM NAC for 6 h (+NAC); after a 3 h pretreatment with NAC, 500 μM iron citrate was added for an additional 3 h (+NAC, +Fe); control experiments were without NAC and Fe (−Fe, −NAC). (**A**) Measurement of iron concentration in NAC-treated, NAC/Fe co-treated and Fe-treated plantlets showing that NAC treatment does not alter their iron loading. Values represent the means of three different experiments and deviations for each point were below 10%. (**B**) Ferritin mRNA abundance visualized on Northern blots using two probes: probe A (Figure 3) corresponding mainly to a part of the coding sequence and probe B (Figure 3) an *AtFer1*-specific probe from the 3′ UTR.

plant hormone ABA [15]; iron loading of maize plants transiently increases their ABA concentration, and, as a consequence, induces RAB gene expression. The involvement of this hormone in the cellular cascade leading to ferritin synthesis in the response of *A. thaliana* to iron was investigated in liquid cultures. Unlike maize [15], an *A. thaliana* RAB gene (namely *RAB18* [21]) is not induced by iron loading treatment (Figure 1B). Furthermore, a 12 h treatment of wild-type seedlings with exogenous ABA did not increase ferritin mRNA abundance (Figure 5), whereas the *RAB18* gene from *A. thaliana* was induced by this hormonal treatment (Figure 5). In addition, ferritin mRNA abundance increased in response to a 12 h treatment with iron of two *A. thaliana* mutants *abi1* and *abi2* which are affected in their response to ABA in vegetative tissues (Figure 5). In conclusion, ABA is not involved in *AtFer1* mRNA accumulation in response to iron overload in the experimental conditions tested.

AtFer1 mRNA accumulation in response to iron is antagonized by the antioxidant NAC

In maize, part of the iron response leading to ferritin mRNA accumulation is independant of the ABA pathway and is mediated via an oxidative pathway which has been shown to be antagonized by co-treatment with NAC [22]. In order to determine whether such an iron-mediated oxidative stress was involved in the increased ferritin abundance in *A. thaliana* seedlings, the influence of an iron/antioxidant co-treatment on *AtFer1* mRNA abundance was determined. NAC treatment had no effect on iron loading of the seedlings, as determined by measuring total iron concentrations (Figure 6A). In contrast, this antioxidant inhibited ferritin mRNA accumulation, demonstrating that an oxidative pathway is involved in this system (Figure 6B). When a probe specific to the *AtFer1* cDNA was used (probe B, 3′ UTR DNA fragment in Figure 3), the corresponding transcript was accumulated in response to iron treatment, and this specific mRNA accumulation was inhibited by the antioxidant treatment (Figure 6).

DISCUSSION

This work describes both the characterization of the first dicotyledonous plant ferritin sequence outside the *Leguminosae* family and, more importantly, the use of an experimental system for the study of iron induction of ferritin synthesis using the model plant *A. thaliana*. The *AtFer1* cDNA is probably incomplete since no polyA$^+$ tail is found at its 3′ extremity and as there are only 38 nucleotides of 5′ UTR (Figure 1), whereas the 5′ UTR of other plant ferritin mRNA is likely to contain an average of 100 bases [8]. Nevertheless, this cDNA was suitable for use as a probe in studies of the regulation of ferritin synthesis in *A. thaliana* in response to iron, and to determine the primary structure of *A. thaliana* ferritin.

Structure/function of *A. thaliana* ferritin

Without N-terminal sequence data for the *A. thaliana* mature ferritin subunit, it is difficult to be certain of the exact transit peptide cleavage position within the *AtFer1* deduced amino acid sequence (Figure 1). However, from sequence comparisons with other plant ferritins (Figure 2) it can be assumed that the chloroplast transit peptide cleavage point is likely to occur at the level of the three Ala residues at positions 48, 49 and 50 found immediately downstream of Val-46 and -47. This position is in agreement with the fact that valine and alanine are very often

found at positions −3 and −1 in signal sequences [39]; the transit peptide cleavage point has been experimentally confirmed in the case of pea and soybean ferritin precursors [5,7]. The *A. thaliana* ferritin subunit, as do other plant ferritin subunits, contains an extension peptide [4,5] of 28 amino acids. This peptide has been suggested to be an important determinant controlling the stability of the protein *in vitro* [41,42], and probably during seed germination [43]. The C-terminal part of this extension peptide contains a three-turn α-helix (the P-helix [5]) which is flanked by two proline residues at positions 16 and 28 and which is probably responsible, in part, for the stabilization function of the extension peptides of plant ferritins [42]; this P-helix and the two proline residues mentioned above are conserved within the AtFer1 protein (Figure 2). Comparison of the mature amino acid sequence common to animal and plant ferritin (i.e. excluding the transit and the extension peptides) revealed that the *A. thaliana* ferritin has the same structural characteristics as other plant ferritin subunits analysed so far. It is more closely related to animal H-type ferritin subunit (48 % identity) than to L-type (36 % identity), and it contains the seven amino acids residues Glu-27, Tyr-34, Glu-61, Glu-62, His-65, Glu-107 and Gln-141 (human-H nomenclature in [44], and italic numbers in Figure 2) of the so-called ferroxidase centre responsible for rapid oxidation of ferrous iron. Ala-13 (human-H numbering in Figure 2), found in place of His-13 in human H ferritin, has been shown to play a role in the ferroxidase activity of plant ferritin [42], and it is conserved in *A. thaliana* ferritin. Additional carboxy residues (Glu-57 and -60, according to human-H numbering in Figure 2), however, are known to be responsible for the superiority of L-chain in nucleation of mineral cores, and have also been described in plant ferritin subunit sequences, leading to the hypothesis that the plant protein was an H/L hybrid; these Glu residues are conserved in the *A. thaliana* ferritin. A peculiarity of plant ferritin is that it contains an E-helix in the C-terminal part of the protein (positions 192–201 in Figure 2 [5]) whose amino acid sequence is conserved in the various plant species analysed so far, but which is divergent from the primary sequence of the E-helix found in animal ferritin. This characteristic is also found in the AtFer1 protein and confirms that channels in the 4-fold-symmetry axis of the 24-mer apoferritin are hydrophilic in plant ferritin and hydrophobic in animal ferritin.

Synthesis of *A. thaliana* ferritin in response to iron overload

Ferritin mRNA and protein accumulate in response to iron in *A. thaliana* seedlings growing in liquid culture (Figure 4), giving the opportunity to study regulation of plant ferritin synthesis in response to iron in this model system. In contrast to maize ferritin expression [8,15], exogenous ABA does not promote the *A. thaliana AtFer1* ferritin mRNA accumulation, at least in this system, although the RAB18 transcript [21] is accumulated in response to this hormonal treatment (Figures 1B and 5). In maize an ABA-independent pathway has also been described which results from an iron-mediated oxidative stress, involving reactive oxygen intermediates; this pathway is antagonized by co-treatment with NAC. The two pathways may operate through differential expression of two different maize ferritin genes named *ZmFer1* and *ZmFer2* respectively, one responding to ABA and the other to the oxidative pathway ([8]; G. Savino, S. Lobréaux and J.-F. Briat, unpublished work). In *A. thaliana*, iron induction of *AtFer1* mRNA accumulation is clearly inhibited by NAC (Figure 6), indicating that the oxidative pathway described in maize is conserved in *A. thaliana*. Our results, therefore, raise the question of the existence, if any, of an ABA-regulated ferritin gene in *Arabidopsis*. If such a gene exists in *A. thaliana*, its coding sequence must be divergent from that of *AtFer1*, as genomic analysis indicates that *AtFer1* is a unique gene (Figure 3). This is a major difference from maize, a monocotyledonous plant, in which both *ZmFer1* and *ZmFer2* genes are very similar in their coding sequence, making discrimination between their respective transcripts impossible when using a cDNA probe corresponding to the coding part of these transcripts [8]. The existence of plant ferritin genes with sequences which are significantly divergent from those reported so far has already been suggested in cowpea, a dicotyledonous plant from the *Leguminosae* family [45]; PCR has established that three cowpea ferritin genes are expressed in leaves and one of these genes shows great divergence from the two others.

We are indebted to Dr. T. Desprez (Laboratoire de Biologie Cellulaire, INRA, Versailles, France) for the gift of the *AtFer1* cDNA, Dr. F. Parcy (Institut des Sciences Végétales, CNRS, Gif sur Yvette, France) for the gift of seeds of the *abi1* and *abi2* mutants and of the RAB18 probe. We acknowledge Dr. H. Logan for careful reading of the manuscript. This work was supported by CNRS (URA 573 and ATIPE grant no. 93N60/0563 to J.-F. B.).

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Received 19 February 1996/12 April 1996; accepted 23 April 1996