

Structure and differential expression of the four members of the *Arabidopsis thaliana* ferritin gene family

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Four ferritin genes are found within the complete sequence of the *Arabidopsis thaliana* genome. All of them are expressed and their corresponding cDNA species have been cloned. The polypeptide sequences deduced from these four genes confirm all the properties of the ferritin subunits described so far, non-exhaustively, from various plant species. All are predicted to be targeted to the plastids, which is consistent with the existence of a putative transit peptide at their N-terminal extremity. They also all possess a conserved extension peptide in the mature subunit. Specific residues for ferroxidase activity and iron nucleation, which are found respectively in H-type or L-type ferritin subunits in animals, are both conserved within each of the four *A. thaliana* ferritin polypeptides. In addition, the hydrophilic nature of the plant ferritin E-helix is conserved in the four *A. thaliana* ferritin

subunits. Besides this strong structural conservation, the four genes are differentially expressed in response to various environmental signals, and during the course of plant growth and development. *AtFer1* and *AtFer3* are the two major genes expressed in response to treatment with an iron overload. Under our experimental conditions, *AtFer4* is expressed with different kinetics and *AtFer2* is not responsive to iron. H_2O_2 activates the expression of *AtFer1* and, to a smaller extent, *AtFer3*. Abscisic acid promotes the expression of only *AtFer2*, which is consistent with the observation that this is the only gene of the four to be expressed in seeds, whereas *AtFer1*, *AtFer4* and *AtFer3* are expressed in various vegetative organs but not in seeds.

Key words: abscisic acid, H_2O_2 , iron, plant, storage.

INTRODUCTION

Ferritins are ubiquitous multimeric iron storage proteins that can store up to 4500 iron atoms in their central cavity, therefore having an essential role in cellular iron homeostasis [1]. In animals, two different types of ferritin subunit, H and L, encoded by different genes have been described. The H subunits contain conserved amino acids defining a ferroxidase site responsible for rapid Fe(II) oxidation, leading to a rapid uptake of iron inside the protein cavity; L subunits lack this site but are enriched in E residues facing the central cavity of the protein, thus enabling better nucleation of Fe(III) for its long-term storage. The relative abundance of each subunit in a native ferritin molecule therefore modulates its function. H-rich molecules achieve the rapid detoxification of iron, whereas a lower uptake rate and long-range iron storage occur in L-rich ferritin molecules [1]. Although a 2–3-fold increase in L-mRNA abundance has been reported to be due to transcriptional control in response to iron overload [2], the major consequence of iron excess is to derepress the translation of both L- and H-mRNA, leading to a 30-fold increase in the abundance of H and L ferritin subunits. This translational control occurs through the iron-responsive element/iron-responsive protein (IRE/IRP) system, which has been widely documented [3,4].

In plants, ferritins have been observed in specific organelles, the plastids, but not in the cytoplasm (as found in animal cells) [5,6]. So far, only one type of plant ferritin subunit has been described, sharing the characteristics of both the H and L subunits, namely a ferroxidase centre and additional E residues facing the protein cavity [7]. Nevertheless, plant ferritins have

been reported to be encoded by a small gene family in maize and *Vigna unguiculata*. Some members have been characterized and shown to be expressed differentially in response to environmental signals or in the course of development [8,9]. In addition, no IRE sequence is found in the 5' untranslated region (UTR) of plant ferritin mRNA or genes characterized so far [10]. Consistent with such a structural observation is a report of the transcriptional control of a soybean ferritin gene in response to iron excess [6]. It has recently been shown that this control was achieved through transcriptional repression for the *ZmFer1* and *AtFer1* ferritin genes from maize and *Arabidopsis thaliana* respectively [11].

However, no exhaustive analysis of all the members of the ferritin gene family in the same plant species has yet been reported. The recent publication of the complete genomic sequence of the model plant *A. thaliana* [12] gave us the opportunity to characterize the organization and structure of all members of the ferritin gene family in the same plant and to start to ascertain their differential patterns of expression in response to various exogenous signals and during the course of plant growth and development.

EXPERIMENTAL

Plant cultures and treatments

All experiments were performed with *A. thaliana* Columbia C24 ecotype. After harvest, all samples were frozen in liquid nitrogen and stored at -70°C .

Plants were cultivated on soil (Humin substrate N2 Neuhaus, Klasmann-Deilmann, Geeste, Germany) in a greenhouse at 23°C

Abbreviations used: ABA, abscisic acid; BAC, bacterial artificial chromosome; EP, extension peptide; EST, expressed sequence tag; IDRS, iron-dependent regulatory sequence; IRE, iron-responsive element; IRP, iron-responsive protein; RT-PCR, reverse-transcriptase-mediated PCR; UTR, untranslated region.

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with a light intensity maintained above 300 $\mu\text{E/s}$ per m^2 and a day/night regime of 16 h/8 h.

Germination of *A. thaliana* seeds *in vitro* was performed in Petri dishes containing half-strength Murashige and Skoog medium [13] with 0.5% agar. Seeds were spread on the nutrient medium and the Petri dishes were stored for 2 days at 4 °C. For germination, plates were transferred to a growth chamber at 24 °C, 16 h/8 h day/night and 300 $\mu\text{E/s}$ per m^2 .

Iron treatments were performed with plants grown hydroponically in a growth chamber at 70% relative humidity, with a light intensity of 400 $\mu\text{E/s}$ per m^2 and a day/night temperature regime of 8 h at 22 °C and 16 h at 20 °C respectively. To support the plants, a polystyrene raft floating on nutrient solution in a 8-litre container was used. Seeds were germinated directly on wet rockwool placed in holes made in the polystyrene raft, thus allowing contact between the nutrient solution and the plants [14]. At 2 weeks after germination the nutrient solution was aerated. The nutrient solution contained the following element concentrations: 1.25 mM KNO_3 , 1.50 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.50 mM KH_2PO_4 , 0.75 mM MgSO_4 , 50 μM Fe-EDTA (Sigma), 50 μM H_3BO_3 , 12 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.7 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1 μM ZnCl_2 , 0.25 μM $\text{MoO}_4 \cdot \text{Na}_2 \cdot 2\text{H}_2\text{O}$ and 0.1 mM $\text{Na}_2\text{O}_3\text{Si}$. The nutrient solution was replaced every week with freshly made solution. Treatments with iron were performed on 5-week-old plants; 1 week before treatment, no iron was added to the nutrient solution. To extract apoplastic iron, roots were washed for 3 min in a 0.3 mM bathophenanthroline disulphonic acid (Sigma) solution containing 5.7 mM dithionite (Merck) and rinsed with distilled water. Iron overload was achieved by the addition of a nutrient solution containing 500 μM iron and 1 mM citrate, prepared as described in [15].

Treatment with H_2O_2 was performed with plants grown *in vitro* in liquid cultures. *A. thaliana* seeds were surface-sterilized by immersion in a 4% (w/v) Bayrochlor (Bayrol)/50% (v/v) ethanol solution for 20 min, with constant shaking. Seeds were washed three times with ethanol and left to dry in sterile conditions. Seedlings were grown in 250 ml Erlenmeyer flasks containing 100 ml of half-strength Murashige and Skoog medium, pH 5.7, and 100 μM Fe(III)-EDTA, supplemented with 1% (w/v) sucrose and 0.5 g/l Mes. After 8 days of culture at 24 °C under continuous light (300 $\mu\text{E/s}$ per m^2) on a rotating table (60 rev./min), plantlets were rinsed with half-strength Murashige and Skoog medium and grown for 7 h in this liquid medium containing 5 mM H_2O_2 (Sigma).

Treatments with abscisic acid (ABA; Sigma) were performed with plants grown hydroponically under sterile conditions. Plants were grown *in vitro* in GA-7 Magenta boxes (Sigma); treatments with ABA were performed as described previously [16].

cDNA cloning and sequencing

Classical molecular biology methods were used as described in [17] except when specified. Alignments were achieved with CLUSTAL W 1.8 [18] and database searches with BLAST [19]. To screen an *A. thaliana* cDNA library [20] for *AtFer3* and *AtFer4* cDNA cloning and in accordance with the expressed sequence tag (EST) sequences, 3'-end-specific primers of *AtFer3* cDNA (primer 1, 5'-ACCCCAAGGACCGTAATAGC-3'; primer 2, 5'-CAGAATCGATATTGATCAAATCTACC-3') and *AtFer4* cDNA (primer 3, 5'-GGTTTGATGAACCTTAT-ACCAGAATATAAAG-3'; primer 4, 5'-GTGATCATGTCTT-TTGTCTCTTACAG-3') were designed. The full-length *AtFer3* and *AtFer4* cDNA species were obtained by nested PCR with the *A. thaliana* cDNA library as a template. Two forward primers carrying the vector arm sequence of pFL61 (primer A, 5'-GCG-

TAAAGGATGGGGAAAGAGAAAAG-3'; primer B, 5'-CAG-ATCATCAAGGAAGTAATTATCTAC-3') were successively used with either the reverse primers 1 and 2 or 3 and 4. Both PCR fragments were cloned and sequenced. A BLASTN search of GenBank® with *AtFer3* and *AtFer4* cDNA sequences permitted the characterization of the corresponding genomic sequences. *AtFer3* and *AtFer4* are respectively on bacterial artificial chromosome (BAC) F18O21 and T07M07 (accession numbers AL163763 and AF085279).

Before *AtFer2* cDNA cloning, the *AtFer2* gene sequence was determined with the use of an inverse PCR approach. *A. thaliana* genomic DNA was digested with *Hind*III and circularized at a concentration of 2 ng/ μl with 0.02 unit/ μl T4 DNA ligase. With ligated DNA fragments as a template, inverse PCR was performed with *AtFer2*-specific primers (primer 5, 5'-CGACA-TTGTCTCTGTCAAAGTAGGCAT-3'; primer 6, 5'-CAAGC-GTGGTGGGAGAGTGAAG-3'). Amplification of a 1.4 kbp genomic fragment was obtained after 35 PCR cycles with Extra-Pol I (Eurobio, Les Ulis, France). The PCR fragment was cloned and sequenced. To clone full-length *AtFer2* cDNA, specific primers spanning the *AtFer2* open reading frame were designed from this genomic sequence (primer 7, 5'-CCTTTCTCTCTC-TAGATCTTAAGCCC-3'; primer 8, 5'-GAAACAGTGGGAC-TTGCAATTTATTATCAGAC-3'). They were used in a reverse-transcriptase-mediated PCR (RT-PCR) assay performed with M-MLV Reverse Transcriptase RNase H Minus (Promega) and Extra-Pol I (Eurobio), in accordance with the manufacturer's instructions. As a template, mRNA species isolated from *A. thaliana* siliques were used. The amplified fragment was cloned and sequenced.

RNA preparation and Northern blot analysis

Total RNA from siliques and dry seeds was extracted as described in [21]; total RNA from leaves, stems, roots and flowers was extracted as described previously [22].

For Northern blot analysis, 10 μg of total RNA for each sample was separated by electrophoresis through a 1.2% (w/v) agarose/formaldehyde gel [23] and blotted on to a nylon membrane (Hybond N; Amersham). Blotted RNA was hybridized with the labelled probes.

Specific probes hybridizing to the 3' UTR of *AtFer1*, *AtFer2*, *AtFer3* and *AtFer4* genes were generated by PCR; 20 ng of DNA was used as a template. The reaction buffer contained 0.25 unit of ExtraPol I (Eurobio), 1 mM MgCl_2 , 333 nM [^{32}P]dATP, 666 nM dATP and the three other dNTPs each at 30 μM . The extension time was set to 3 min; 45 cycles were performed. The sequences of the primers used were 5'-ACTTGGACCTC-TCTAAGTTCAC-3' and 5'-CTTCATTGATTAAGCTTACG-AAG-3' for the *AtFer1* probe, 5'-GGAAGGAGAGTTCAGC-TTCTGAG-3' and 5'-GAAACAGTGGGACTTGCAATTTAT-TATC-3' for the *AtFer2* probe, 5'-TCTGAGTTTTAGCTGT-AATCTCGC-3' and 5'-ACCCCAAGGACCGTAATAGC-3' for the *AtFer3* probe, and 5'-GCAGCTTCGAGGGACTC-TTGTGTC-3' and 5'-GGTTTGATGAACCTTATAACCAGA-ATATAAAG-3' for the *AtFer4* probe.

EF1- α , GST6, RAB18 and 25 S rRNA DNA probes were labelled with [^{32}P]dCTP with the use of Prime-a-Gene Labelling kit (Promega).

Hybridizations were performed overnight at 42 °C in the presence of 50% (v/v) formamide. After being washed twice for 5 min with 2 \times SSC [300 mM NaCl/30 mM trisodium citrate (pH 7.0)] containing 0.1% SDS at 22 °C and twice for 15 min with 0.1 \times SSC/0.1% SDS at 42 °C, filters were autoradiographed at -70 °C with X-Omat films (Kodak) or with a

phosphor screen and a PhosphorImager (Storm; Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RESULTS

A. thaliana genome contains four expressed ferritin genes

We have previously reported the cloning and characterization of an *A. thaliana* ferritin cDNA and its corresponding gene named *AtFer1* [11,16]. Analysis of the *A. thaliana* EST database with BLASTN revealed four groups of ferritin sequences, indicating that these proteins are encoded by a gene family composed of at least four members (Table 1). Accession numbers for the largest EST of each ferritin gene available at the beginning of this study are indicated in Table 1. Sequence analysis of these ESTs enabled us to design specific deoxyoligonucleotides for PCR screening of a cDNA library from *A. thaliana* plantlets. This approach led to the cloning of *AtFer3* and *AtFer4* full-length cDNA species, with sizes of 1053 and 1063 bp respectively. Genomic sequences corresponding to *AtFer3* and *AtFer4* mRNAs were recovered by searching GenBank; the corresponding BAC numbers are indicated in Table 1. For *AtFer2* a genomic DNA fragment bearing 429 bp of the 5' untranslated sequence and 990 bp of the proximal part of *AtFer2* were recovered by inverse PCR. Deoxyoligonucleotides designed from this genomic sequence and from the *AtFer2* EST sequence (Table 1) led to cloning of the full-length *AtFer2* cDNA by RT-PCR on mRNA species from *A. thaliana* siliques. A GenBank search with this sequence enabled us to recover the complete sequence of the *AtFer2* gene on BAC F9F8 (Table 1). Computer analysis of the complete *A. thaliana* genome sequence reveals that ferritin is encoded by four genes corresponding to the *AtFer1-AtFer4* cDNA species described above.

The four *A. thaliana* genes encoding ferritins have identical organizations in terms of numbers and positions of introns. The seven introns in each gene are located at precisely the same place as reported for two maize ferritin genes and one soybean ferritin gene [8,24]. This confirms the original intron/exon organization of plant ferritin genes in comparison with animal counterparts, which contain only three introns located at different positions from those for plant ferritins. As regards chromosome mapping, *AtFer1* gene is localized on chromosome 5, *AtFer2* and *AtFer3* are on chromosome 3 and *AtFer4* is on chromosome 2 of *A. thaliana* (Table 1).

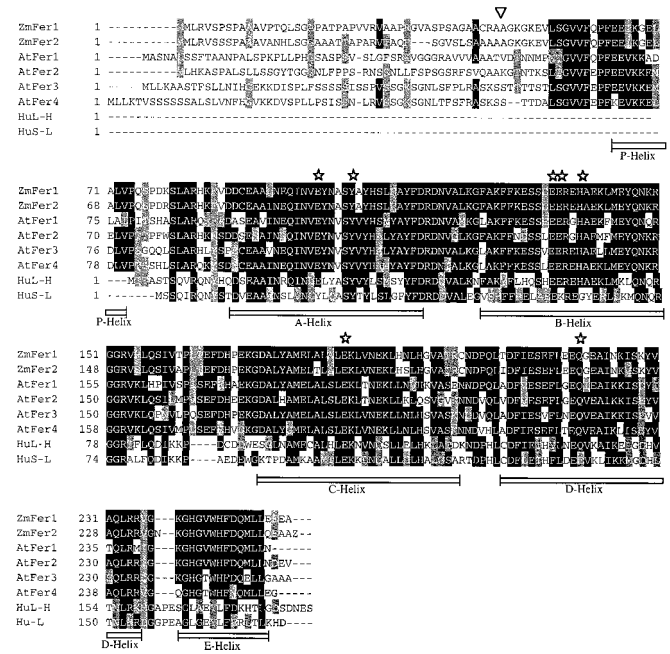
Amino acid sequence comparison of the four A. thaliana ferritin subunits

The *AtFer1* protein sequence deduced from *AtFer1* cDNA has already been reported [16]. Cloning and sequencing of the three other cDNA species of this gene family enabled us to deduce the amino acid sequence of the corresponding ferritin subunits and to perform an amino acid sequence alignment of the four

Table 1 Database information related to the Arabidopsis thaliana ferritin gene family

Ferritin gene	GenBank® accession number of the longest EST	cDNA accession number	BAC (accession number)	Chromosome
<i>AtFer1</i>	Z18109	X94248	(AF229850)	V
<i>AtFer2</i>	Z34949	AJ312192	F9F8 (AC009991)	III
<i>AtFer3</i>	T76041	AJ312190	F18O21 (AL163763)	III
<i>AtFer4</i>	Z30895	AJ312191	T07M07 (AF085279)	II

A



B

	<i>AtFer1</i>	<i>AtFer2</i>	<i>AtFer3</i>	<i>AtFer4</i>	<i>ZmFer1</i>	<i>ZmFer2</i>	<i>HuL-H</i>	<i>HuS-L</i>
<i>AtFer1</i>	100%	--	--	--	--	--	--	--
<i>AtFer2</i>	71.6%	100%	--	--	--	--	--	--
<i>AtFer3</i>	66.3%	71.6%	100%	--	--	--	--	--
<i>AtFer4</i>	70.9%	74%	77.8%	100%	--	--	--	--
<i>ZmFer1</i>	66.8%	58.1%	69.2%	72.6%	100%	--	--	--
<i>ZmFer2</i>	62.6%	69.9%	67.9%	70.8%	95.2%	100%	--	--
<i>HuL-H</i>	47.3%	46.8%	44.6%	48.4%	48.9%	48.4%	100%	--
<i>HuS-L</i>	37.6%	36.1%	39.9%	39.6%	39.1%	38.5%	53.6%	100%

Figure 1 Amino acid sequence comparison of various ferritin subunits

(A) Plant ferritin subunit precursors (*Zea mays*, *ZmFer1* and *ZmFer2*; *A. thaliana*, *AtFer1*, *AtFer2*, *AtFer3* and *AtFer4*) and human ferritin subunits (*HuL-H* and *HuS-L*) were aligned with the CLUSTAL W 1.8 program. Identical residues are boxed in black and similarities are indicated in grey. The positions of the predicted helices are shown. Stars indicate amino acids known to be involved in the ferroxidase centre of human H ferritins. A white triangle indicates the position of the cleavage site of the transit peptide of *ZmFer* proteins as determined by N-terminal sequencing of the mature subunit [22]. (B) Percentage identities of the amino acid sequences compared in (A).

A. thaliana ferritin subunits (Figure 1A). Our present knowledge indicates that plant ferritin subunits are synthesized as precursors with an N-terminal transit peptide responsible for plastid targeting of the subunit, where they assemble as 24-mers after cleavage of the transit peptide [25]. The amino acid sequence deduced from the four *AtFer* cDNA species revealed that they all contain an N-terminal extension upstream of a sequence region that is highly conserved with animal ferritin sequences (Figure 1A). The first part of this extension presents all the characteristics of a plastid transit peptide [26]. Furthermore, amino acid sequence analysis of the four *AtFer* polypeptides, with ChloroP software [27], predicts their targeting to the plastids, in agreement with the localization of the plant ferritin protein reported previously [10,25].

The four mature ferritin subunits possess a plant-specific sequence named extension peptide (EP), which is observed in other plant ferritin subunit sequences reported previously. This

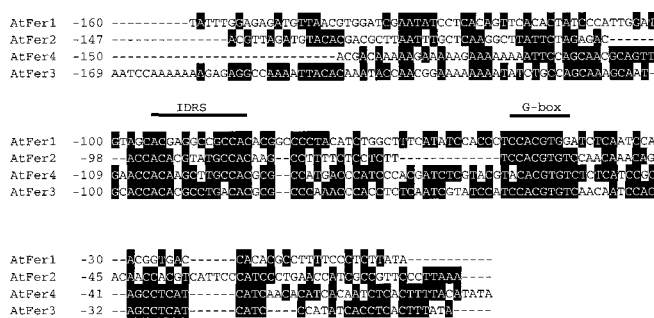


Figure 2 Sequence alignment of the proximal region of *A. thaliana* ferritin gene promoters

AtFer promoter sequences were aligned with the CLUSTAL W 1.8 program [18], starting at the putative TATA box sequence. The IDRS region involved in *AtFer1* regulation by iron is indicated by an overline [11]. The G-box element conserved in all promoter sequences is also indicated.

EP sequence has been reported to have a role in the control of protein stability [28]. The two proline residues flanking the P α -helix, predicted in the three-dimensional model structure of the pea seed ferritin subunits [7], are conserved in the four *A. thaliana* EP sequences. Sequence conservation in this region indicates that its structure is likely to be conserved in the various plant ferritin subunits.

Mature ferritin subunits from *A. thaliana* share more than 70% sequence identity, except *AtFer1* and *AtFer3*, which share 66.3% identity (Figure 1B). This strong overall conservation was also observed when *A. thaliana* ferritin subunit sequences were compared with maize ferritin subunit sequences as representatives of monocotyledonous plants; identities range from 58% between *ZmFer1* and *AtFer2* to 72.6% between *ZmFer1* and *AtFer4* (Figure 1B). When compared with human ferritin subunit sequences, *AtFer* subunits shared approx. 50% identity with the H-type subunit and almost 40% with the L-type subunit, confirming previous results indicating that plant ferritin subunit sequences are more related to H-type animal ferritin subunits than to L-type subunits. This statement is reinforced by the fact that all *AtFer* subunits contain, as do other plant ferritins, conserved amino acids necessary for ferroxidase activity, a typical feature of animal H-type ferritin [1]. However, like all plant ferritin subunits characterized so far, *AtFer* subunits also contain the conserved E residues that are present in L-subunits and are responsible for better nucleation and iron core stability; the exception is Glu-64, which is replaced by a glycine residue in *AtFer1* and *AtFer2* (Figure 1); the impact of this change, if any, on ferritin function is currently unknown. Finally, the E helix present at the COOH extremity of all eukaryotic ferritin subunits is also found in the *AtFer* subunits; its amino acid sequence is identical with that of other plant ferritin subunits but different from the sequence observed in animal ferritin subunits.

Selected features of *AtFer* promoter sequences

It has been reported previously that expression of the various members of plant ferritin gene family could be differentially regulated in maize or in *Vigna unguiculata* [8,9], with part of this regulation occurring at least at the transcriptional level. This suggested that some of the molecular determinants of this differential expression could be found within the promoter regions of the various ferritin genes. At least one of these determinants was recently characterized functionally in the promoter sequences of the *ZmFer1* and the *AtFer1* ferritin genes from

maize and *A. thaliana* respectively. It consists of a 15 bp *cis*-element named iron-dependent regulatory sequence (IDRS) that is necessary for the transcriptional repression of *ZmFer1* and *AtFer1* under conditions of iron deficiency [11]. Characterization of the four expressed ferritin genes from *A. thaliana* (Table 1) prompted us to compare their promoter sequences. The IDRSs identified in *AtFer1* and *ZmFer1* share some similarities with the same region of the *AtFer2*, *AtFer3* and *AtFer4* promoter sequences (Figure 2). However, it remains to be determined whether or not the IDRS-like sequences observed in the *AtFer2*, *AtFer3* and *AtFer4* promoter sequences are functional. In addition, a putative *cis*-element involved in iron regulation has been described in a soybean ferritin gene promoter [29]; no such element is present in any of the four *A. thaliana* genes. A putative G-box (positions -40 to -48 in Figure 2), described in *ZmFer1* and *AtFer1* promoter sequences as unnecessary for the transcriptional regulation of these genes by iron [11], is nevertheless conserved in the promoter region of the three other members of the *A. thaliana* ferritin gene family (Figure 2). G-boxes are known to act synergistically with other *cis*-regulatory elements, in particular for genes regulated during embryogenesis and/or in response to the plant hormone ABA [30].

Differential expression of the four *AtFer* genes in response to treatments with iron, ABA and H₂O₂

In maize, two ferritin genes (*ZmFer1* and *ZmFer2*) have been shown to be regulated differentially [8]. Both genes are actively transcribed under conditions of iron excess but with different kinetics and through independent pathways. *ZmFer2* is regulated through an ABA-dependent pathway, whereas the regulation of *ZmFer1* by iron is ABA-independent and requires the IDRS mentioned previously [11]. *ZmFer1* expression is also induced by treatment with H₂O₂ but this pathway does not require a functional IDRS [11,31].

Our knowledge of the four *AtFer* gene sequences (Table 1) prompted us to design specific 3' UTR probes to measure the abundance of each transcript in response to treatments with iron, ABA and H₂O₂. The sequences of the four probes, obtained by PCR with specific deoxyoligonucleotides (see the Experimental section) shared only 45–49% identity. Before their use in Northern blot experiments, their specificity was tested by hybridization of each probe to the four *AtFer* cDNA species. Each probe specifically recognized its cDNA (results not shown).

Total RNA prepared from roots or leaves of *A. thaliana* plantlets treated with excess iron were analysed by Northern blotting with the various *AtFer* probes described above (Figure 3). The abundance of *AtFer1* mRNA increased in roots and leaves in response to treatment with iron. This increase was observed in both roots and leaves as early as 3 h after the beginning of treatment. A difference in the kinetics of accumulation of *AtFer1* mRNA occurred between roots and leaves, a maximum being reached in leaves after 6 h of treatment, whereas the abundance continued to increase in roots at the 12 h time point. The abundance of *AtFer3* mRNA increased with a very similar kinetics to that of *AtFer1* mRNA in response to iron. *AtFer2* mRNA was detected at a very low level and its quantity was not up-regulated in response to iron. *AtFer4* mRNA was not detected in roots but its abundance was clearly increased in leaves in response to iron, although with different kinetics from that observed for *AtFer1* and *AtFer3* mRNA species, the maximum being observed at the 12 h time point. RNA blot hybridizations with a 25 S rRNA probe indicated that the same amount of RNA was loaded in each lane.

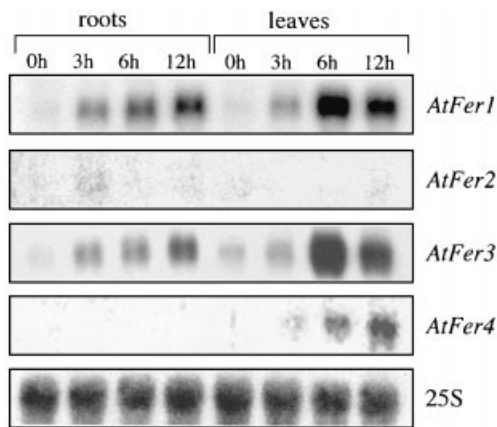


Figure 3 Accumulation of ferritin mRNA in response to overloading of *Arabidopsis* plants with iron

Iron-starved *A. thaliana* plants 6 weeks old, cultivated hydroponically, were treated or not with 500 μM iron citrate for 3, 6 or 12 h. RNA extracted from roots or leaves was analysed by Northern blotting and hybridized with the indicated gene-specific probes that specifically recognized AtFer1, AtFer2, AtFer3 or AtFer4 mRNA. The 25 S probe hybridized to 25 S rRNA and was used as a control of the total RNA quantity (10 μg) loaded in each lane.

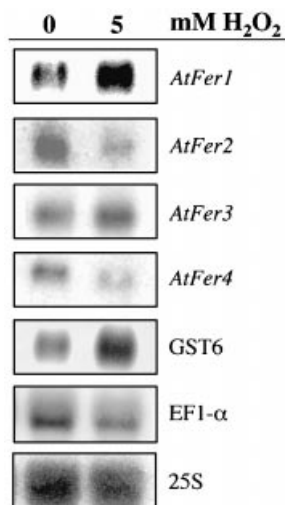


Figure 4 Accumulation of ferritin gene transcript in response to treatment of *Arabidopsis* plantlets with H_2O_2

A. thaliana plantlets 8 days old, cultivated *in vitro* in a liquid medium, were treated or not with 5 mM H_2O_2 for 7 h. RNA extracted from plantlets was analysed by Northern blotting with the indicated AtFer gene-specific probes. The GST6 cDNA probe was used as a H_2O_2 -inducible control and the EF1- α cDNA probe as a non- H_2O_2 -inducible control. The 25 S probe hybridized to 25 S rRNA and was used as a control of the total RNA quantity (10 μg) loaded in each lane.

Northern-blot analysis, with the same probes, of total RNA from *A. thaliana* plantlets treated for 7 h with 5 mM H_2O_2 revealed a 3-fold increase in the abundance of AtFer1 mRNA (Figure 4). AtFer3 mRNA was also slightly more abundant in response to the treatment, whereas the abundance of AtFer2 and, to a smaller extent, AtFer4 transcripts was decreased in response to treatment with H_2O_2 . Hybridization of these RNAs with a glutathione S-transferase probe was used as a positive control for treatment with H_2O_2 [32]; hybridization with an EF1- α probe was used as a negative control. Hybridization of the

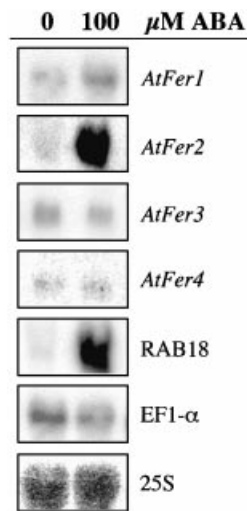


Figure 5 Accumulation of ferritin gene transcript in response to treatment of *Arabidopsis* plants with ABA

A. thaliana plants 2 weeks old, cultivated *in vitro*, were treated or not with 100 μM ABA for 24 h. RNA extracted from plants was analysed by Northern blotting and hybridized with the indicated AtFer gene-specific probes. The RAB18 cDNA probe was used as an ABA-inducible control and the EF1- α cDNA probe was used as a non-ABA-inducible control. The 25 S probe hybridized to 25 S rRNA and was used as a control of the total RNA quantity (10 μg) loaded in each lane.

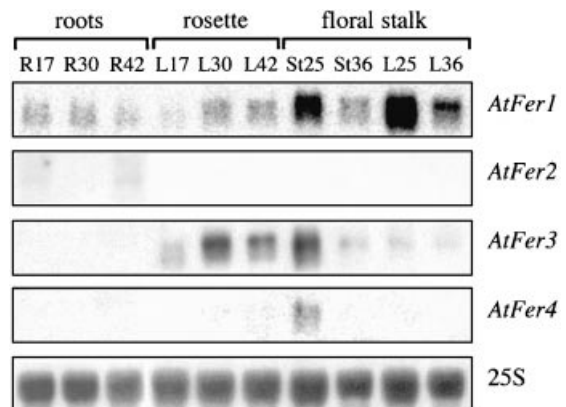


Figure 6 Ferritin mRNA abundance during development, in roots, leaves and stems of *Arabidopsis* plants

A. thaliana plants were grown on soil in a greenhouse. RNA was isolated from roots (R), leaves (L) or stems (St); numbers indicate the age (in days) of plants when organs were collected. RNA extracted from the different organs was analysed by Northern blotting and hybridized with the indicated probes specific for AtFer1, AtFer2, AtFer3 and AtFer4. The 25 S probe hybridized to 25 S rRNA and was used as a control of the total RNA quantity (10 μg) loaded in each lane.

blots with a 25 S rRNA probe indicated that the same amount of RNA was loaded in each lane.

Total RNA species from ABA-treated *A. thaliana* plantlets were also analysed. Only the abundance of AtFer2 mRNA was strongly increased in response to this hormonal treatment; the transcript levels of AtFer3 and AtFer4 seemed insensitive to treatment with ABA (Figure 5). The abundance of AtFer1 mRNA was very slightly increased in response to treatment with ABA, which could have been due to a secondary effect related to oxidative stress. As a positive control, the level of RAB18

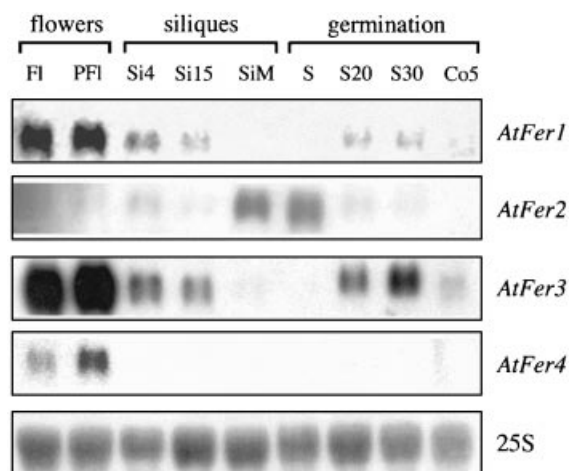


Figure 7 Accumulation of ferritin gene transcript during development, in flowers, siliques and during germination of *Arabidopsis* plants

A. thaliana plants were grown on soil in a greenhouse or *in vitro* for the samples collected during germination. RNA was isolated from flowers before pollination (FI) or after pollination (PFI), siliques [Si; numbers indicate the age (in days) of siliques when collected], mature siliques (SiM), seeds (S, dry seeds; S20, S30, seeds after being soaked in water for 20 or 30 h respectively) or plantlets 5 days old with cotyledons (Co5). RNA extracted from the different organs was analysed by Northern blotting and hybridized with the indicated *AtFer* gene-specific probes. The 25 S probe hybridized to 25 S rRNA and was used as a control of the total RNA quantity (10 µg) loaded in each lane.

mRNA, encoded by an ABA-responsive gene [33], was shown to increase strongly in response to treatment with ABA. The abundance of EF1- α mRNA was unaffected by treatment with ABA.

Differential expression of the four *AtFer* genes during the course of plant growth and development

It has been reported that ferritin synthesis and degradation are regulated, both spatially and temporally, during plant development. In pea, the protein accumulates during seed formation, is degraded during the early stages of germination and is not observed in vegetative organs [34]. At the RNA level, a semi-quantitative RT-PCR approach permitted a description of the differential expression of various ferritin genes in different organs of *V. unguiculata* [9].

With the use of the various ferritin cDNA probes described above, we performed a Northern blot analysis of total RNA species prepared from various *A. thaliana* organs harvested at various time points during their growth and development (Figures 6 and 7). As already observed in response to exogenous signals, the abundance of *AtFer1* and *AtFer3* mRNA species fluctuated similarly during plant development, although with some specificities in vegetative organs. Both *AtFer1* and *AtFer3* mRNA species were observed in rosette leaves, their abundance being higher at days 30 and 42 than at day 17. In roots, *AtFer1* mRNA had the same abundance at the three time points considered, whereas *AtFer3* mRNA was almost undetectable. In floral stalks, *AtFer1* and *AtFer3* mRNA species were observed in the stem, with a maximum at day 25 in comparison with day 36; however, *AtFer1* mRNA was detected abundantly in leaves from the floral stalk with a maximum at day 25, whereas *AtFer3* mRNA was detected only weakly, with no variation between the two time points considered (Figure 6). Both *AtFer1* and *AtFer3* mRNA species accumulated strongly in flowers before or after

pollination and were still detected in 4-day-old and 15-day-old siliques; however, they were not observed in mature siliques or dry seeds. Both *AtFer1* and *AtFer3* mRNA species re-accumulated during the early stages of germination, after 20 or 30 h of seed imbibition and in 5-day-old germinating plantlets showing cotyledons (Figure 7).

The expression pattern of *AtFer2* mRNA during *A. thaliana* development was markedly different from those observed for *AtFer1* and *AtFer3* mRNA species: it was almost undetectable in roots, rosette or floral stalk leaves, stem, flowers, immature siliques or germinating seeds, at all time points considered (Figures 6 and 7). In contrast, *AtFer2* mRNA was abundantly present in mature siliques and dry seeds, whereas other *AtFer* mRNA species were not detected (Figure 7).

The expression pattern of *AtFer4* mRNA diverged significantly from the two types of pattern reported above for *AtFer1* and *AtFer3* mRNA species on the one hand and for *AtFer2* mRNA on the other hand. *AtFer4* mRNA was observed only in floral stalk at day 25 (Figure 6) and in flowers, with a maximum after pollination (Figure 7).

DISCUSSION

Complete sequencing of the *A. thaliana* genome gave us, for the first time, the opportunity to describe all members of the ferritin gene family of a plant. Sequence analysis of the four genes and of the corresponding cDNA species and the deduced amino acid sequences of the subunits allow us to discuss some points related to plant ferritin structure, subcellular localization and biochemical function. First, all of the genes of the plant ferritin family contain seven introns at precisely conserved positions (results not shown; see the accession number in Table 1), which establishes a major difference between the plant and animal kingdoms and confirms specific evolutionary constraints suggested previously [24]. Secondly, no IRE was observed in any 5' UTR of the four *AtFer* mRNA species (results not shown; see the accession number in Table 1), proving that the IRE/IRP system *sensu stricto*, which is involved in the translational control of animal ferritin mRNA species [3,4], is not conserved in plants for the control of ferritin gene expression in response to excess iron. Thirdly, the four amino acid sequences deduced from the *AtFer* cDNA species we have cloned and sequenced reveal a N-terminal extension in all cases when compared with animal ferritin subunit sequences (Figure 1). The first part of this extension shares characteristics with plant-specific transit peptides responsible for the targeting of precursor proteins to plastids [26]. Although no experimental demonstration is currently available, this structural observation strongly suggests that all plant ferritin subunits encoded by the four *AtFer* genes are located within plastids. This is in agreement with electron microscopy studies and immunocytological and biochemical results that demonstrate the presence of this protein in this subcellular compartment in many plant species [10]. The second part of this N-terminal extension is conserved in the four ferritin subunits and shares similarities with previously described extension peptides [28]. This suggests that the function(s) associated with this plant-specific domain, in particular that connected with protein stability control, are conserved in all ferritin subunits found in a plant. Finally, although the percentages of identity of the four mature plant ferritin subunits are closer to H-type than L-type animal ferritin subunits (Figure 1B), they all share H-type and L-type animal characteristics in terms of the ferroxidase site and the glutamic residues involved in the nucleation process. Therefore a biochemical functional specialization of ferritin, as occurs in animal

systems with the existence of H and L heteromers bearing various ratios of the two subunits, is questionable in plants.

Sequence determination of the four members of the *A. thaliana* ferritin gene family enabled us to design 3' UTR-specific probes for each transcript. It allowed a first exhaustive study of the differential expression, at the RNA level, of all the ferritin genes of a plant, in response to key signals known to be involved in plant ferritin gene expression, such as iron, H₂O₂ and ABA [25], and in various organs at different stages of plant growth and development. In response to treatment with excess iron, we confirmed an increase in the abundance of AtFer1 mRNA, and extended this to AtFer3 and AtFer4 mRNA species (Figure 3). It has recently been reported that a 15 bp *cis*-regulatory element, found both within the *AtFer1* promoter and the maize *ZmFer1* promoter, was, at least in part, necessary for the iron-regulated expression of these genes [11]. Such an IDRS sequence is partly conserved in the *AtFer3* and *AtFer4* promoters and it would be important to determine whether or not it is functional in the response to iron. With regard to the activation of ferritin gene expression by H₂O₂, the fact that the *AtFer1* gene is positively regulated by this treatment (Figure 4) is an additional argument that this *A. thaliana* gene is the orthologue of maize *ZmFer1* and is in agreement with the report that both genes have their expression inhibited by antioxidants [16,30]. It is currently unknown why *AtFer2* and *AtFer4* genes seem slightly down-regulated in response to treatment with H₂O₂ (Figure 4).

AtFer2 is the only *A. thaliana* ferritin gene that is responsive to treatment with exogenous ABA (Figure 5). In this respect it is reminiscent of the regulation of the maize *ZmFer2* gene by ABA [8]. However, the latter was also responsive to treatment with iron, which was not observed with *AtFer2*. This discrepancy between the two plant systems remains to be explained. Nevertheless, the response of *AtFer2* to exogenous ABA is in agreement with its major expression in mature siliques and in dry seeds (Figure 7), where it is the only *AtFer* gene to be expressed. Indeed, it is well established that ABA-responsive genes often encode seed-specific proteins [35]. It is also known that under non-stress conditions most of the ferritin protein is found within the seeds, where it accumulates [34]. Furthermore, 92% of iron found in seed embryos is contained within ferritin molecules [36]. In addition, although the *AtFer4* gene has been described on BAC T07M07, which contains a cluster of ABA-regulated genes [37], our results demonstrated that it is not up-regulated in response to treatment with exogenous ABA (Figure 5). In *A. thaliana* it is therefore very likely that most of the seed ferritin proteins result exclusively from the expression of *AtFer2*. *AtFer2* can also be detected in vegetative organs of plantlets when grown *in vitro* in liquid cultures (Figure 4) and in response to treatment with ABA (Figure 5), suggesting a role for this gene in response to stress in these organs. Ferritin gene expression in vegetative organs (roots, leaves, stems) during *A. thaliana* growth and development is achieved mainly by *AtFer1* and *AtFer3*, which share many expression territories (Figures 6 and 7). This suggests that these two genes account for ferritin synthesis in young plantlets, in senescing leaves, in the floral stalk and in flowers and young siliques. Consistent with our results is the observation that a ferritin cDNA specifically expressed during the senescence of *Brassica napus* leaves has been described [38]. It is also important to notice that *AtFer1* is the only ferritin gene expressed in roots (Figure 6). This result is in agreement with a report showing that only one ferritin gene is expressed in *V. unguiculata* roots [9]. The pattern of expression of *AtFer4* is highly restricted, to flower stalks and flowers (Figures 6 and 7). Future experiments allowing tissue and cellular localization of *AtFer4* gene expression will be required, to gain an insight into its role in these organs.

This study indicates that four expressed ferritin genes are necessary and sufficient to maintain this aspect of plant iron homeostasis in *A. thaliana*. No major structural differences can be predicted from the amino acid sequences of each subunit deduced from these four genes. However, important expression specificities are observed for each gene, indicating that the control of their differential expression is likely to be a key determinant of their biological roles. These roles could be investigated by studying the effect on the phenotype, at physiological and biochemical levels, of mutations in each *A. thaliana* ferritin gene.

We thank Karine Mouline (BPMP, Montpellier, France) for the gift of RNA samples from ABA-treated plantlets and from siliques, and Sébastien Léon (BPMP) for a critical reading of the manuscript. We acknowledge the *Arabidopsis* Biological Resource Center from the Ohio State University for providing ferritin ESTs.

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Received 26 June 2001/25 July 2001; accepted 29 August 2001