Ferritins and Iron Storage in Plants

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Review

Ferritins and iron storage in plants

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ABSTRACT

Iron is essential for both plant productivity and nutritional quality. Improving plant iron content was attempted through genetic engineering of plants overexpressing ferritins. However, both the roles of these proteins in the plant physiology, and the mechanisms involved in the regulation of their expression are largely unknown. Although the structure of ferritins is highly conserved between plants and animals, their cellular localization differ. Furthermore, regulation of ferritin gene expression in response to iron excess occurs at the transcriptional level in plants, in contrast to animals which regulate ferritin expression at the translational level. In this review, our knowledge of the specific features of plant ferritins is presented, at the level of their (i) structure/function relationships, (ii) cellular localization, and (iii) synthesis regulation during development and in response to various environmental cues. A special emphasis is given to their function in plant physiology, in particular concerning their respective roles in iron storage and in protection against oxidative stress. Indeed, the use of reverse genetics in *Arabidopsis* recently enabled to produce various knock-out ferritin mutants, revealing strong links between these proteins and protection against oxidative stress. In contrast, their putative iron storage function to furnish iron during various development processes is unlikely to be essential. Ferritins, by buffering iron, exert a fine tuning of the quantity of metal required for metabolic purposes, and help plants to cope with adverse situations, the deleterious effects of which would be amplified if no system had evolved to take care of free reactive iron.

1. Introduction

Iron in plants is essential for specific functions such as photosynthesis, making it one of the key elements required for CO_2 fixation and therefore for biomasss production. This process leads to O_2 production, which can react with iron to produce reactive oxygen species which can be ultimately deleterious for the cell integrity. Iron homeostasis needs therefore to be strictly controlled to avoid deficiency and toxicity, which are both known to dramatically impair the physiology of plants, affecting consequently their development and growth.

Homeostasis of iron in plants is achieved through very dynamic processes requiring proteins and small organic molecules in order to take up the metal from the soil, to traffic it throughout the plant, to compartmentalize it intracellularly, and ultimately to buffer and to store it in case of excess.

Among the molecules required in these processes, the ferritins are a class of ubiquitous iron storage proteins, found in all living kingdoms. The purpose of this review is to present an overview of our current knowledge of the specific features of plant ferritins, at the level of their (i) structure/function relationships, (ii) cellular localization, and (iii) synthesis regulation during development and in response to various environmental cues. A special emphasis is given to their function in plant physiology, in particular concerning their respective roles in iron storage and in protection against oxidative stress.

2. Specific features of plant ferritins

2.1. At a structure/function level

It has been known for almost 20 years that plant and animal ferritins have evolved from a common ancestor gene as suggested by amino acid sequence comparison of ferritin subunits [1]. Indeed, plant ferritin subunit sequences share between 39% and 49% identity with mammalian ferritin sequences. The 7 amino acids involved in the ferroxidase center of the animal H type ferritins, allowing an oxidation and a rapid uptake of iron inside the mineral core are found in plant ferritin subunits [2,3]. Despite this higher identity of the plant ferritins with H type animal ferritins, carboxylic residues found at the surface of the cavity of L-type animal ferritins, and responsible for an efficient iron nucleation and a better stability of the mineral core, are also conserved in plant ferritins. Ferritin subunits are synthesized as a 32-kDa precursor which contains a unique N-terminal sequence consisting of two domains absent in animal ferritins. The first domain, around 40–50 residues long, is the transit peptide involved in the

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transport of ferritin subunit precursor into the chloroplast, and likely the mitochondria (see Section 2.2; Fig. 1). The second domain is part of the mature protein, and named extension peptide. Its function could be related to protein stability, and it could be cleaved during the germination process of pea seeds through free radical damages [4–6]. The 30 carboxy-terminal amino acids are highly conserved within plant ferritins but they have diverged from their animal counterparts. In animal ferritin, conserved amino acids from this domain define the fifth or E-helix which is involved in the formation of hydrophobic channels located at the 4-fold symmetry axes (Fig. 1).

A very high level of similarity of the secondary structure of pea seed ferritin with mammalian ferritin secondary structure has been reported [7]. A high helical content is consistent with the adoption by residues 41–210 of a 4-helix bundle conformation (helices A, B, C, and D in Fig.1). A non-helical stretch of 21 residues (positions 106–127 in pea seed ferritin) showed either turn/coil or b-strand predictions and would link the B and C helices via an L-loop characteristic of the animal ferritin subunit fold. Interestingly, the prediction indicated the presence of an additional 3-turn α-helix in the N-terminal extension region of the protein. This helix of 11 amino acids in length would be found lying within the 24 amino acids plant specific sequence. A further consequence of the secondary structure as predicted is the conservation of the short C-terminal E-helix, despite the divergent primary structures of plant and animal ferritins in this region as noted above.

Using the coordinates of the recombinant human H ferritin structure [8], a model of the three dimensional structure of pea seed ferritin subunit has been proposed (Fig. 1) [7]. This model superimposed on the known subunit structure of recombinant human H ferritin, highlighting the remarkable conservation of the 3-D core structure of plants as modelled to human ferritins, and the predicted regions of change. At the N-terminal extremity of the pea subunit, nearly half of the additional residues from this specific extension region would assume a helical conformation which folds back onto the surface of the subunit (Fig.1). This extension peptide is the site for free radical cleavages during iron exchanges *in vitro* [9], and possibly *in vivo* [5], leading to ferritin degradation (Fig. 1). Mutagenesis analysis has confirmed the role of the extension peptide in plant ferritin protein stability [6]. The model also supports the presence of: (i) the E-helix at the same place in plant and animal ferritins and (ii) a ferroxidase center in the plant ferritin subunit.

The *Arabidopsis thaliana* genome which is fully sequenced [10] contains four ferritin genes (*AtFer1–4*). At the protein level, the immunodetection of ferritin subunits by western blot of crude protein extracts from leaves and seeds of various ferritin knock-out mutants from this model plant has recently been achieved [11] (Fig. 2). It makes it possible to conclude that only FER1 exists as a 28-kDa mature subunit in leaves, and that it is the more expressed ferritin at the protein level in this organ. It is also deduced from these experiments that FER3 and FER4 would exist in leaves only as a 26.5-kDa processed subunit. Finally, the only ferritin subunit present in seeds, as a 26.5-kDa subunit, is encoded by the *AtFer2* gene, which is consistent with the expression data recorded at the transcript level. It is therefore interesting to notice that *Arabidopsis* ferritin subunits can be observed, as in pea [5,9] and in soybean [12] as two polypeptides of different molecular weights. Sequencing of purified assembled ferritin from soybean seeds enabled the identification of two subunits, H-1 and H-2 [12]. H-2 is an unprocessed 28-kDa subunit, whereas H-1 exhibited a

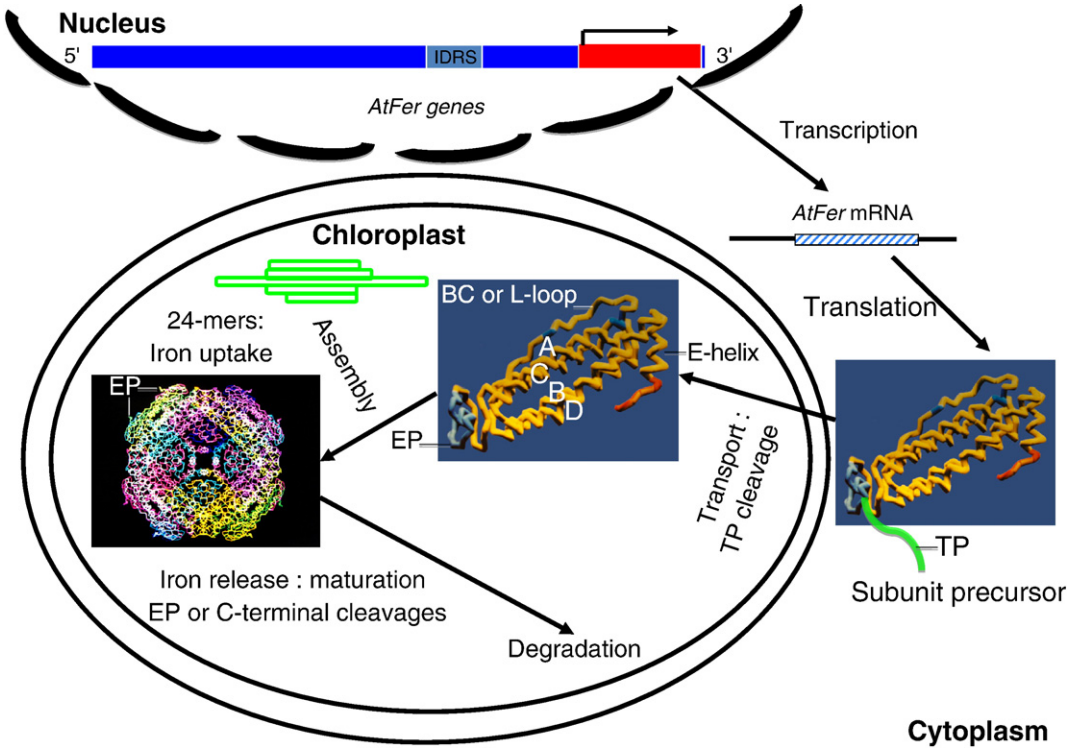

Fig. 1. "Ferritin cycle" in plant cells. Ferritins are encoded by nuclear genes regulated by iron, through *cis*-element(s) such as the Iron Dependent Regulatory Sequence (IDRS). Ferritin mRNA translation leads to the synthesis of ferritin subunit precursors, containing a N-ter extension composed of two parts: (i) the extension peptide (EP) and (ii) the transit peptide (TP), responsible for chloroplast targeting of ferritin subunit, and which is cleaved during the transport of the precursor through the chloroplast enveloppe. The plant ferritin subunit structure superimposed to the human H ferritin subunit structure ±1 Å. A bundle of four α-helices (A, B, C, and D) forms the bulk of this structure. The L-loop links the B and C helices and is involved in the dimerisation of ferritin subunits prior to their assembly as 24-mers. The E-helix forms a 60° angle with the A–B–C–D bundle and is involved in the formation of channels at the 4-fold symmetry axis of the assembled protein. The EP plant specific sequence forms an additional α-helix of 3 turns, which is observed at the surface of the assembled 24 subunits mature ferritin. The assembled 24-mers ferritin stores Fe(III) in its central cavity. Iron release from the assembled protein promotes its maturation through N-ter cleavages within the EP, or C-ter cleavages (see Figs. 2 and 3), ultimately leading to ferritin protein degradation. The color code for ferritin subunit structure is as follow: green is for the transit peptide found at the N-ter extremity of the subunit precursor; yellow indicates the conservation with ferritin animal structure; blue is for the EP plant specific N-ter sequence and for a faint modification in the L-Loop; and red indicates a region downstream of the E-helix which is not conserved with the animal ferritin subunit structure.

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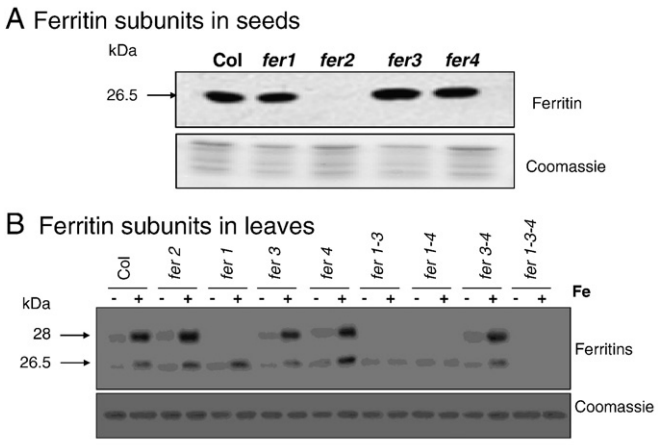

C *fer2* and *fer1-3-4* mutant phenotypes in various organs

	Leaves	Stem	Flowers	Seeds
fer 2	-	-	-	- Susceptibility to oxidative stress during germination
fer 1-3-4	- Biomass decrease under high iron - Decreased CO_2 fixation	- Decreased Fe content - Altered expression of metal transporter genes	- Increased Fe content - Sterility under high iron - Increased ROS production - Increased activities of ROS detoxifying enzymes - Altered expression of metal transporter genes	- Decrease in seed yield under high iron

Fig. 2. Ferritin subunits in *Arabidopsis thaliana* and ferritin mutant phenotypes. *Arabidopsis* ferritin are encoded by 4 genes, *AtFer1* to *AtFer4* [59]. The isolation and characterization of knock-out mutants in the 4 genes [11] allowed to determine the molecular mass of the corresponding proteins, the localization of the subunit expression, and the responsiveness to exogenous iron application. Ferritin subunits were detected using the serum raised against FER1 protein [11,68]. A: ferritin accumulation in seeds. Total extracts from Col, and from *fer1*, *fer2*, *fer3*, and *fer4* (knock-out mutants in *AtFer1*, *AtFer2*, *AtFer3* and *AtFer4* respectively) were used. FER2 subunit is the only one expressed in seeds. B: ferritin accumulation in leaves. Three week-old plantlets were treated with water (−) or 500 μM Fe-citrate (+). Leaves were collected 5 h later, and total protein extracts were used to detect ferritin subunits. The simple mutants *fer1*, *fer3* and *fer4* were crossed to obtain *fer1-3*, *fer1-4* and *fer3-4* double mutants. The triple mutant *fer1-3-4* was obtained by crossing *fer1-3* and *fer1-4*. Leaf ferritin consist of two different subunits of 28 and 26.5 kDa. The analysis of the different mutants shows that *AtFer1* encodes the 28 kDa subunit, and that *AtFer2*, *AtFer3* and *Atfer4* encode 26.5 kDa subunits. C: phenotypes of the *fer2* and *fer1-3-4* mutant plants observed in leaves, stem, flowers and seeds.

C-terminal truncation of 17 residues leading to the obtention of a 26.5-kDa polypeptide. Cleavage occurs after an arginine residue, absent in H-2 (Fig. 3). Alignment of the C-terminal part of the four *Arabidopsis* ferritin subunits with H-1 and H-2 soybean forms shows that the arginine residue at the cleavage site of H-1 is present in FER2, FER3 and FER4 subunits, but not in FER1 (Fig. 3). This result is consistent with observed molecular masses of the *Arabidopsis* ferritin subunits (Fig. 2). Interestingly, the alignment of the C-terminal parts of algae, moss and higher plant ferritins shows that only some dicot ferritin subunits contained a leucine or methionine residue instead of the arginine (Fig. 3), suggesting that the unprocessed ferritin subunits are specific to dicotyledonous plants. The biological and functional significance of the presence of cleaved and uncleaved ferritin subunits has been studied in soybean both *in vivo* and *in vitro*. First, native soybean and pea seed ferritins are heteromultimers consisting of the two H-1 and H-2 subunits [12,13]. The obtention of homo- and heteromultimers of soybean ferritin subunits *in vitro* [12,14] indicates that the control of different subunit stoechiometries in the native 24-mer protein could lead to native proteins with different iron oxidation and release properties. This is reminiscent of the situation observed in animals, where the stoechiometry between H and L subunits led to native ferritins with different functional properties. However, the biological function of the plant subunit heteromultimerization remains to be assessed *in vivo*.

Ferritin subunits assemble in a compact 24-mer arrangement with 4.3.2 symmetry. The resulting packed protein shell contains many intersubunit interactions except that at the four-fold and three-fold symmetry axes, channels of about 3A wide are formed (Fig. 1) [2,3]. The predicted structure of plant ferritins has a similar quaternary structure. The plant specific N-terminal extension mentioned above would form a compact localized domain on the external surface of the 24-mer. In animals, the 4-fold channels are hydrophobic while the 3-fold channels are hydrophilic. The plant 3-fold channels would remain hydrophilic, and the 4-fold channels would be hydrophilic. In animals, hydrophobic channels at 4-fold axes present an energy barrier to unshielded Fe^{2+} ions while the hydrophilic 3-fold channels have been considered as a possible entry or exit pore, and a site for Fe(II) oxidation. The consequences of both 4-fold and 3-fold channels being hydrophilic and the effects this would have on iron exchange in plant ferritins remain to be determined.

The nucleation and growth of the iron core are active biomineralization processes involving functions of the protein coat [2]. The predicted ferroxidase activity of plant ferritins has been confirmed experimentally either with ferritin purified from pea seeds [15] or with recombinant pea seed ferritin expressed in *Escherichia coli* [16]. However, this activity was found to be lower than that of the recombinant human H ferritin; though, it was much more active than the recombinant human L-ferritin. Furthermore, when Ala-13 was replaced by His in recombinant pea seed ferritin, as in mammal ferritins, the ferroxidase activity was significantly reduced [6]. Ferrous iron, the substrate of the ferroxidase activity, may be formed *in vitro* by reduction with ascorbate of exogenous ferric chelates and/or of ferric ferritin-iron itself [17]. Photoreduction of ferric citrate and/or of ferritin-iron may also be a source of ferrous iron [18]. Depending on the concentration of ascorbate either an overall iron release (ascorbate>2.5 mM) or uptake (ascorbate<2.5 mM) by pea seed ferritin may occur. *In vitro* studies of the different steps of these independent and simultaneous uptake and release fluxes have revealed that uptake was faster at pH 8.4 than at pH 7 or 6 and was inhibited by an excess of free iron-binding ligands. pH values and ligand/Fe ratios fit known physiological values. These free ligands were also found to be inhibitory during ferritin-iron release. The *in vitro* conditions mentioned above are likely to occur *in vivo*, indicating that the redox state of plastids could be the main driving force for controling iron exchange between the ferritin cavity and the environment of the protein.

Not only does the plant ferritin structure have some pecularities, but the mineral core hosted in the central cavity of the protein also deserve attention. Mineral cores of animal ferritins and of bacterioferritins have significant chemical and structural differences as revealed by electron microscopic appearance and Mössbauer spectroscopy behavior [2]. Human and horse ferritin molecules contain a single domain crystal of ferrihydrite, associated with variable amounts of inorganic phosphate. Bacterioferritin cores are hydrous ferric phosphate, with high amounts of phosphate, having a poor cristallinity and very low magnetic ordering temperatures. These physico-chemical differences between animal and bacterial iron cores can be attributed to variations in phosphate concentration between bacteria and animal cells [19]. Plant ferritins are proteins of eukaryotic type found in a prokaryotic type environment (i.e. the plastids and eventually mitochondria). Such a situation influences the composition and the structure of plant ferritin cores as determined by measurement of Fe:P ratio, and by Mösbauer spectroscopy, electron microscopy and X-ray absorption spectroscopy analysis of native and

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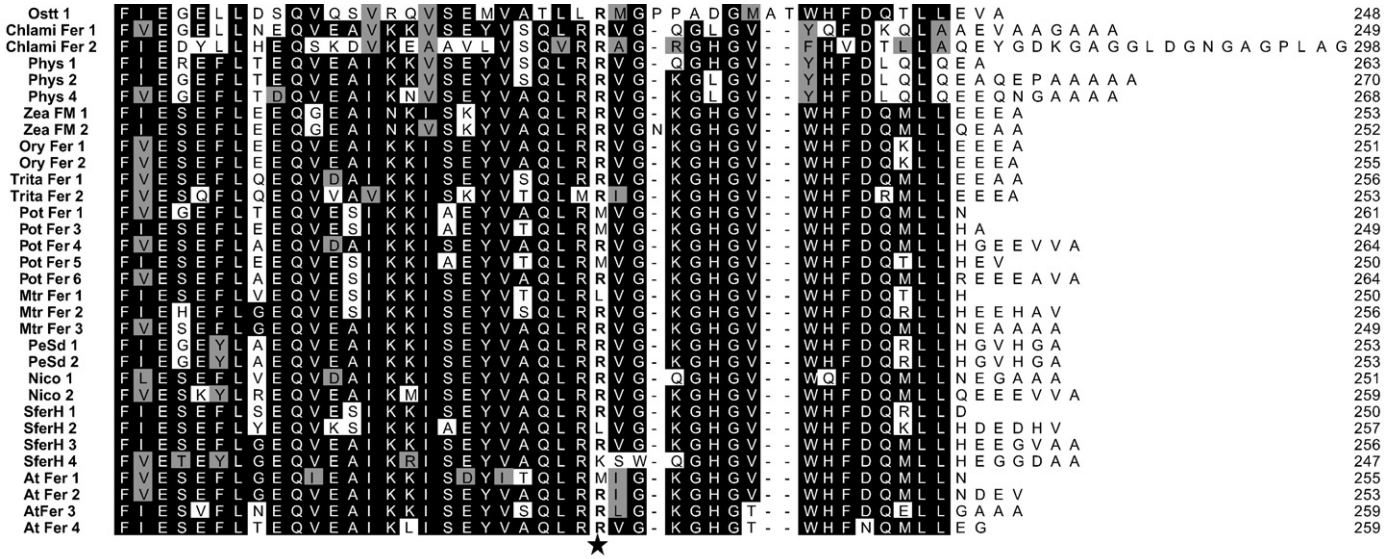

Fig. 3. Sequence alignment of the C-terminal parts of some plant ferritin subunits. Identical and similar residues with soybean (*Glycine max*) and *Arabidopsis* subunits are boxed in black and grey, respectively. Amino acids are numbered from the translational start methionine. The arginine in position 234 of soybean H-1 subunit was shown to be the last residue of the processed 26.5-kDa form [12].This residue and the corresponding arginines in other subunits are indicated in bold, and positioned by a star. Sequences come from green algae (*Ostreococcus tauri*: Ostt1; *Chlamydomonas reinhardtii*: ChlamiFer1 and ChlamiFer2), moss (*Physcomitrella patens*: Phys1, Phys2, and Phys4) monocots (*Zea mays*: ZeaFM1, and ZeaFM2; *Oryza sativa*: OryFer1 and OryFer2; and *Triticum aestivum*: TritaFer1 and TritaFer1), and dicots (*Popolus trichocarpa*: PotFer1, PotFer3, PotFer4, PotFer5, and PotFer6; *Medicago truncatula*: MtrFer1, MtrFer2, and MtrFer3; *Pisum sativum*: Pesd1 and Pesd2; *Nicotiana tabacum* Nico1 and Nico2; *Glycine max*: SferH1, SferH2, SferH3, and SferH4; and *Arabidopsis thaliana*: AtFer1, AtFer2, AtFer3, and AtFer4). Proteins were named, and sequences recovered from [69].

reconstituted pea seed ferritin mineral cores [15,20]. Plant ferritin mineral cores have high phosphate content and are amorphous. In contrast, reconstituted mineral cores using pea seed apoferritin in the absence of phosphate were crystalline ferrihydrite. Plant mineral cores are therefore more related to their bacterial counterpart than to their animal one, and this is likely to be due to the high phosphate concentration found within organelles, in comparison to the cytosol.

2.2. At a cellular level

It was suggested 40 years ago that plants may contain ferritins [21], and the first characterization of these protein in plants has been reported 10 years later [22]. Ferritins have been described in almost all plant species and have been detected in many cells by classical electron microscopy methods. A major difference between animal and plant ferritins concerns their subcellular localization. Whereas ferritins are mostly cytosolic soluble proteins in animal cells, they are mainly found in the stroma of various plastids inside the plant cells; although described in chloroplasts, ferritins have been mainly observed in non-photosynthetic plastids such as proplastids, etioplasts, chromoplasts and amyloplasts [23]. Biochemical confirmation of the plastid localization of plant ferritin was obtained by showing that the subunit of the protein synthesized in response to iron treatment of bean leaves, or of soybean cells, was synthesized as a precursor with a molecular weight slightly higher than that of the mature subunit found inside the chloroplasts after its translocation (Fig 1.); this ferritin precursor was taken up *in vitro* by purified chloroplasts [24–27]. Fig. 1) [28]. Such a transit peptide has been characterized for plant ferritins by comparing the NH_2 terminal amino acid sequence of purified mature subunit [4,29,30] with the amino acid sequences deduced from the nucleotide sequences of various plant ferritin cDNAs [7,29–32].

In mammals, in addition to ferritin genes encoding H- and L-cytosolic subunits of this protein, an intronless gene has been reported to encode a ferritin with a long N-terminal extension of 56–60 amino acids containing a mitochondrial localization sequence (review in [3]). The product of this gene was specifically taken up by mitochondria and processed to form a stable ferritin protein with ferroxidase activity and was functional in incorporating iron. The amino acid sequence of mitochondrial ferritin (FtMt) is highly conserved among the mammals and invariably contains the residues involved in the ferroxidase center. In humans, FtMt has been identified in specific organs and cells such as in testis, and spermatozoa. Structure–function studies of human FtMt revealed remarkable similarities with the cytosolic H ferritin properties, except for a lower ferroxidase activity. The role of mammal FtMt seems to be related to protection of the mitochondria against iron toxicity and oxidative damage. Evidence for localization of plant ferritin in mitochondria in addition to plastids has also been recently documented [33]. It was shown by these authors that a protein of 25–26 kDa cross-reacting with a polyclonal antibody raised against pea seed ferritin was present within a protein extract of pea or *A. thaliana* mitochondria purified through discontinuous percoll gradients. Purification of this 25–26 kDa soluble protein by immunoprecipitation and determination of its primary structure by Mass Finger Print experiments using MALDI-TOF mass spectrometry confirmed it was ferritin. This result was reinforced by the fact that ferritin was observed by immunogold labelling in mitochondria from etiolated pea stem cross-sections or in isolated pea mitochondria. The four ferritin sequences from *A. thaliana* all possess at their N-terminus a typical transit peptide pre-sequence for chloroplast targeting of some nucler encoded proteins. However the analysis of the pre-sequence of AtFer4 reveals a high score for a potential mitochondrial localization, especially when compared with AtFer1. Nevertheless, no functional data have been reported so far demonstrating the presence of the AtFer4 ferritin in *A. thaliana* mitochondria. It was therefore concluded by Zancani et al. [33] that some of the plant ferritin could be dual targetted to both plastids and mitochondria.

2.3. At a gene expression level

As mentioned above, ferritins are localized in different cellular compartments in plants and animals. This specific compartmentation of plant ferritins within plastids suggests that some plant specific pathways would be involved in the regulation of the ferritin subunit and mRNA levels in this living kingdom.

Iron starvation of plants leads to the chlorosis symptoms, resulting from chlorophyll deficiency and impaired photosynthesis. Under such

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conditions, root iron uptake systems are induced in order to enhance iron acquisition and to maintain a physiological integrity [34]. Then, addition of an excess of iron in the culture medium of iron starved plantlets leads to a large iron influx into the plant [30]. This iron is translocated within 3 h into the leaves to restore the essential photosynthetic process in chloroplasts [35]. During this period of regreening which takes about 24 to 48 h, ferritins are used as a safe iron buffer. During this period of recovery from iron deficiency, ferritin mRNA is already detectable in maize leaf plantlets 3 h after iron resupply. This accumulation reaches its maximum respectively 6 and 24 h after the treatment in leaves and roots, and then gradually decreases [36]. This increase in ferritin mRNA abundance preceeds the accumulation of ferritin subunits, with a maximum detected 24 h after the beginning of the iron treatment. Then, a gradual decrease of ferritin content is observed, consistent with a transient iron buffer function of this protein during the regreening of plants [30]. It has been demonstrated that iron resupply to iron starved soybean cell suspension cultures induces a ferritin mRNA accumulation controled at the transcriptional level [26]. This result was confirmed for *AtFer1*, the *Arabidopsis* ortholog of the maize *ZmFer1* gene [31]. It has been shown that both *AtFer1* and *ZmFer1* iron-dependent gene expressions required a *cis*-regulatory element named IDRS (Iron Dependent Regulatory Sequence) [37], which is required for the repression of expression of these genes under iron deficient conditions. A different iron-dependent *cis*-regulatory element than the IDRS, named FRE (Fe Responsive Element), has also been characterized in the promoter region of a soybean ferritin gene [38]. So far, no *trans*-acting factors interacting with these *cis*-regulatory sequences have been characterized. It appears therefore clearly that plant ferritin expression in response to iron excess is regulated at the transcriptional level, in contrast to animal ferritins, the regulation of which occuring mainly at the translational level. This translational control in animal cells requires the IRE/IRP-ACO system [39,40]; the animal cytosolic ACO (aconitase) can switch to the RNA-binding protein IRP1 (iron-regulatory protein1) for binding to the IRE (Iron Responsive Element) found in the 5′ UTR of the ferritin mRNA in order to repress its translation under low iron conditions. Another IRP (IRP2) has been characterized. It has not the ability to switch to an aconitase form because it is unable to accomodate a Fe–S cluster. It is nevertheless regulated by iron, being degraded through the proteasome pathway under low iron conditions. Three IRP1 homologues have been identified in the genome sequence of *A. thaliana*, named ACO1–3 [41]. To determine whether or not they may encode functional IRP1-like proteins and regulate iron homoeostasis in plants, loss-of-function mutants have been obtained for the three genes. The *aco1-1* and *aco3-1* mutants show a clear decrease in cytosolic ACO activity. However, none of these mutants is affected in respect of the accumulation of ferritin transcripts or protein in response to iron excess. *cis*-acting elements potentially able to interact with IRPs have been searched in the *Arabidopsis* genome. They appear to be very rare sequences, found in the 5′-UTR or 3′-UTR of a few mRNAs unrelated to iron metabolism. They are therefore unlikely to play a functional role in the regulation of iron homoeostasis. These results confirm that in plants, the cytosolic ACO-IRP1/IRE regulatory system is not conserved for regulating iron homoeostasis.

Despite the growing number of physiological conditions reported to date leading to plant ferritin synthesis (reviewed in [34,42]), little is known about the regulatory molecules acting downstream of iron for the regulation of ferritin expression. Since the *AtFer1* gene is the most expressed ferritin gene in response to iron excess, it was used as a model to study the signaling pathway leading to its iron regulation in an *Arabidopsis* cell culture system [43]. Iron excess and oxidative stress, mimicked by exogenous H_2O_2 application, promote *AtFer1* gene expression through two independent and additive pathways. By combining pharmacological and imaging approaches in this *Arabidopsis* cell culture system, several elements in the signal transduction pathway leading to the increase of *AtFer1* transcript level after iron treatment were identified. NO quickly accumulates in the plastids after iron treatment, it acts downstream of iron and upstream of a PP2A type phosphatase to promote an increase of *AtFer1* mRNA level. The *AtFer1* gene transcription has been previously shown to be repressed under low iron conditions with the involvement of the *cis*-acting element IDRS identified within the *AtFer1* promoter sequence. Experimental data showed that the protein ubiquitinated and degraded in response to iron treatment is unlikely a *trans*-acting factor able to directly bind to the IDRS under low iron conditions (Fig. 4). It is worth noticing that in animal cells, one of the ferritin *trans* regulators involved in the translational repression of ferritin mRNAs in low iron conditions, namely IRP2, is regulated by NO and ubiquitination. After iron addition, IRP2 has been shown to be nitrosylated and subsequently ubiquitinated, and degraded by the proteasome, thus leading to ferritin mRNA translation [44–47]. It is therefore remarkable that molecular effectors of the response to iron excess, such as NO and ubiquitination, are conserved between the translational regulation of animal ferritins and the transcriptional regulation of plant ferritins.

In a recent study, a LUC-based genetic screen, using the *AtFer1* iron-responsive promoter as a target, led to identify TIME FOR COFFEE (TIC) as a negative regulator of the *AtFer1* gene expression. In the corresponding mutant, *AtFer1* mRNA was over-accumulated when compared to wild-type, suggesting that TIC is a repressor of the *AtFer1* gene expression [48]. TIC was previously described as a nuclear regulator of the circadian clock in *Arabidopsis* and is specific to plants [49,50]. *AtFer1* gene expression was shown to be clock-regulated, and it is down-regulated in *cca1*, *lhy*, and *elf 4* mutant plants altered in the functioning of the central oscillator of the circadian clock (Fig. 4). Other iron-overload-responsive genes such as *AtFer3*, *AtFer4* and *AtAPX1* are also regulated by TIC and by the circadian clock whereas iron starvation responsive genes (i.e. *AtIRT1*; *AtFRO2*) are not. Interestingly, mutants in *TIC* gene exhibited a chlorotic phenotype rescued by exogenous iron addition, and are hypersensitive to iron during the early stages of development. These results show that the regulation of iron homeostasis in plants is a major output of TIC- and circadian clock-dependent signaling pathways. The IDRS *cis*-element, located in the proximal region of the *AtFer1* promoter and mediating *AtFer1* repression [37] is not involved in the *TIC* regulatory pathway [48].

Thus, *AtFer1* expression is under the control of two independent repressive pathways, one involving the IDRS *cis*-element and controlling the dark-induced transcriptional activation [51], and one involving TIC and controlling the circadian clock-dependent *AtFer1* regulation.

TIC expression is constitutively expressed among the circadian time [49], but *AtFer1* expression is not all the time repressed by TIC. TIC appears to be inactive at dawn, when *AtFer1* mRNA accumulation peaked. This raises the question of the regulation of TIC activity. The screening for TIC interactors led to the isolation of the SNF1-related kinase (SnRK) AKIN10 [52], which was proposed as a master metabolic sensor [53]. AKIN10, and its homologue AKIN11, were shown to interact with SKP1/ASK1 (S-phase kinase associated protein 1/*Arabidopsis* SKP1-like 1) that mediates proteasomal binding of an ubiquitin ligase [54]. This interaction is inhibited by PRL1 (Pleiotropic Response Locus 1) that seemed to compete with SKP1 for binding to the C-terminal regulatory domain of AKIN10 and AKIN11. A recent genetic screen for identifying genes involved in singlet oxygen signaling pathway [55] led to the isolation of a mutant in the *PRL1* gene. Interestingly, the characterization of the *prl1-5* mutant showed that the expression of *AtFer1* and *AtAPX1* was constitutively repressed in this mutant when compared to wild-type [56]. This result suggests that PRL1 could be a positive regulator of the TIC activity. Thus a regulatory network involving TIC, AKIN10 and PRL1 could be hypothesized for regulating iron homeostasis (Fig. 4). When active, TIC would repress the *AtFer* and *AtAPX1* genes. This active state would be (directly or indirectly) promoted by the AKIN10 kinase. Since AKIN10 is negatively regulated by PRL1, AKIN10 may constitutively

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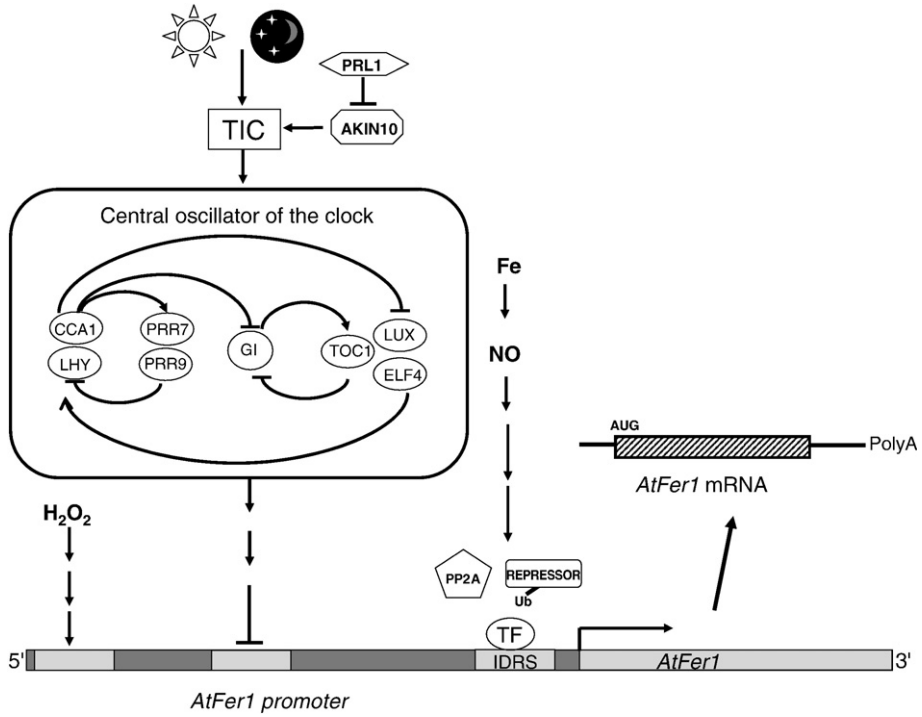

Fig. 4. Model of *AtFer1* transcriptional regulation. This model presents the current knowledge and hypothesis concerning the various factors involved in *AtFer1* regulation. Iron and hydrogen peroxyde positively regulate *AtFer1* transcription by two independant and additive pathways. Under low iron conditions, the *cis*-acting element IDRS (Iron Dependent Regulatory Sequence) is involved in *AtFer1* repression. Upon iron addition, the *AtFer1* gene is de-repressed. This de-repression involves the ubiquitinilation (Ub) and the subsequent 26S proteasome-dependent degradation of a repressing factor (repressor). (De)phosphorylation events are involved in *AtFer1* response to iron, since a PP2A type phosphatase (PP2A) appears to be a positive regulator of *AtFer1*. Iron addition leads to a quick nitric oxide (NO) production in the plastids. This NO production is necessary to *AtFer1* regulation, and acts upstream of the PP2A and the degradation of the repressor in this pathway. *AtFer1* is also transcriptionally regulated by the central oscillator of the circadian clock and by TIME FOR COFFEE (TIC). In mutants of genes encoding components of the central oscillator of the circadian clock (i.e. *cca1*, *lhy* and *elf4*), *AtFer1* circadian regulation is lost. TIC is a repressor of *AtFer1* expression, and acts in a pathway independent of the IDRS, which requires day/night cycling to be active. TIC interacts with the AKIN10 kinase (*Arabidopsis* SNF1 kinase homologue) in two-hybrid experiments. AKIN10 is negatively regulated by PRL1. In *prl1* mutants, *AtFer1* expression is strongly down-regulated. Thus TIC, AKIN10, and PRL1 could be hypothesized to act in the same pathway leading to the regulation of *AtFer1* expression. This model was established with results from [37,43,48,52,54,56]. CCA1: circadian clock associated 1; LHY: late elongated hypocotyl; PRR7 and 9: pseudo response regulators 7 and 9; GI: gigantea; TOC1: timing of cab expression 1 ; ELF4: early flowering 4; LUX: lux arrhytmo.

activate TIC in *prl1* mutants, leading to a lack of *AtFer* and *AtAPX1* de-repression at dawn, and to a constitutive repression.

TIC emerges therefore as a major regulator of iron homeostasis in plants. Interestingly, two integrators of stress and energy signaling AKIN10 and PRL1 are connected to TIC [48,56] and/or involved in the regulation of some iron-responsive gene expression. In response to energy deficit associated with stresses, the SnRK1 kinases seem to initiate genome-wide transcriptional changes that allow to restore homeostasis and to develop long-term responses contributing to adaptation and preservation of growth and development. Regulatory mechanisms were evidenced to coordinate several inputs, such as light, circadian clock or nutrient signals, into a complex signaling network which is just being deciphered. Considering the essential functions of iron and iron-containing proteins in central metabolic processes such as photosynthesis, respiration, nitrogen and sulfur assimilation, interplays regulating iron homeostasis should be assessed and elucidated to understand how the cellular energy signaling is fully integrated into whole plant adaptation and regulation of growth and development. It is very likely that plant ferritins, by buffering iron, exert an appropriate control of the quantity of metal required for metabolic purposes. Therefore, they help plants to cope with adverse situations, the deleterious effects of which would be amplified if no system had evolved to take care of free reactive iron.

3. Respective roles of ferritins and vacuoles in iron storage in plants

The primary function of animal ferritins inside cells is to store iron, and to deliver the metal for metabolism when needed. In bacteria, ferritin function appears to be more related to the protection against oxidative stress, by preventing iron to react with oxygen. In plants, most of the hypothesis formulated regarding ferritin functions were only based on correlations between localization of the proteins and responses of their expression to environmental factors and developmental stages (reviewed in [34,42]). In seeds, ferritins were widely proposed to be the major iron storage form, and to release and provide their metal to the Fe-containing proteins after germination. Seed ferritins would be, therefore, essential for a proper building of the photosynthetic apparatus, and as such would be involved in the autotrophy/heterotrophy transition [5]. In leaves, it was hypothesized that ferritin would be an iron source at early stages of development for the synthesis of iron-containing proteins involved in photosynthesis [5,57,58].

Direct evidences regarding ferritin functions in plants were recently reported through a loss-of-function approach in *Arabidopsis*. Among the 4 ferritin genes from *Arabidopsis*, *AtFer2* is the only one expressed in seeds, whereas *AtFer1*, *AtFer3* and *AtFer4* are expressed in vegetative and reproductive tissues [59]. Knock-out mutants lacking either the seed isoform FER2, or the three isoforms in vegetative tissues, FER1, 3 and 4 were isolated and characterized [11]. FER2 is the only subunit accumulated in dry seeds, and is degraded during germination. However, in *fer2*, total iron content in seeds was not altered compared to wild-type. Iron contained in ferritin was estimated to represent no more than 5% of the total seed iron. Thus, it appears that ferritin does not constitute the major iron storage form in *Arabidopsis* seed. Furthermore, iron stored into ferritin is not essential for the development of the seedling, even under iron limiting conditions. Ferritins appear therefore to have only a minor iron storage

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function in *Arabidopsis*. This statement can be viewed as contradictory to the results obtained *in vitro* with pea seed ferritin and as having demonstrated the role of pH, ascorbate and iron-ligand on iron release by pea seed ferritin [17]. However, although the amount of iron stored in *Arabidopsis* seeds is low relative to total seed iron, this buffered iron is likely to be remobilized, and the physico-chemical parameters defined *in vitro* could be relevant *in vivo*. It is also important to remember that in *Arabidopsis* seeds the bulk of iron is stored into vacuoles complexed to globoids. These globoids are much less abundant in pea seed vacuoles, and iron-ferritin can represent up to 92% of total iron in pea embryo axis. Therefore, there clearly exist differences between species in their way to manage iron in their seeds (see discussion section in [60]). In *Arabidopsis*, the importance of vacuoles in seed iron storage, and its remobilization during germination, has been documented at a molecular level by characterizing the activities of vacuolar NRAMP3 and NRAMP4 efflux transporters and VIT1 influx transporter [60,61]. It prompted the analysis of *AtFer2* ferritin gene expression in different genetic backgrounds affected in iron homeostasis of plastid or vacuolar compartments (*fer*, *nramp* and *vit* knock-out mutants, and *NRAMP* and *VIT* overexpressors) [62]. These studies revealed that ferritin stability in seeds depends on a proper allocation of iron from vacuole to plastid, highlighting a potential cross-talk between the vacuolar and plastidial seed compartments for iron store allocation. These results highlight an integrative response regarding iron homeostasis at the cellular level. Furthermore, from a biotechnological point of view, they indicate that the success of ferritin over-expression strategies for iron biofortification [63,64] would be highly dependent of the control of the mechanisms enabling the translocation of high amount of iron into seed plastids.

4. Ferritins and oxidative stress in plants

Functions of ferritin in leaves, were studied by using a triple *fer1-3-4* mutant, devoid of ferritins in this organ [11]. Under water irrigation, growth of Col and of *fer1-3-4* was similar. When irrigated with iron, the increased biomass observed for Col indicates that this metal was a limiting factor, and was not toxic. By contrast, the *fer1-3-4* growth was reduced, likely due to a decrease of CO_2 fixation (Fig. 2C). In mature leaves, photosynthesis was not affected by the absence of ferritin. The observation that the electron flux through PSII was not different in the mutant and in Col under iron or water irrigation suggests that the absence of ferritins in the triple mutant did not have a severe impact on the photosynthetic electron transfer machinery. However, the decrease of CO_2 fixation observed suggests that the photosynthetic electron transfer chain was less efficiently used by the Calvin cycle enzymes in the absence of ferritins. Pleiotropic defects in flowers were observed when *fer1-3-4* was grown under iron irrigation, leading to a strong reduction of fertility (Fig 2C). These alterations were associated with an increase in the total iron content in the flowers, and a decrease in the stem, suggesting an alteration of iron transport and allocation between these organs (Fig. 2C). Indeed, a transcriptomic analysis of a set of potential iron-transporter genes revealed that the expression of many of them was modified in *fer1-3-4* compared to Col, when grown under elevated iron conditions. The absence of ferritins in reproductive organs therefore strongly alters iron homeostasis and deregulates iron transport between organs, finally leading to alterations of fruit development probably due to an excess iron toxicity. Indeed, the absence of the FER2 ferritin in seeds led to a higher sensitivity to methylviologen, a pro-oxidant compound, as observed during the germination process (Fig. 2C). In leaves and flowers, even under non-iron excess feeding conditions (i.e. irrigation with water), the absence of ferritins in the *fer1-3-4* mutant line led to an increased activity of several reactive oxygen species (ROS) detoxifying enzymes and to an enhanced ROS production, as measured by dichloro-acetate fluorescein imaging (Fig. 2C). Nevertheless, the oxidized and reduced ascorbate and glutathione pools in the mutant were similar to those of Col, and ROS-associated damages were avoided, since lipid peroxidation was not enhanced. These results indicate that, under water irrigation, *fer1-3-4* plants are able to compensate and bypass the lack of safe iron storage into ferritins by increasing the capacity of ROS detoxifying mechanisms. However, when the plants were irrigated with high amounts of iron, the lack of ferritins in *fer1-3-4* plants led to pleiotropic defects both in vegetative and reproductive organs, which strongly impaired plant growth and fertility. It appears that under high-iron conditions, the free iron-associated ROS production overwhelmed the scavenging mechanisms activated in *fer1-3-4*. It is therefore clear that ferritin function in plants is essential for iron sequestration to avoid oxidative stress.

Indeed, the best demonstration that light, photoynthesis, oxidative stress and ferritin synthesis were linked was obtained by a series of studies using *Chlamydomonas reinhardtii* as a model. Two genes, *FER1* and *FER2*, encoding ferritin subunits were characterized in this algae [65]. Both ferritin subunits are plastid localized, but they do not co-assemble. The ratio of ferritin1 to ferritin 2 is 70:1 in iron-replete cells, suggestive of a more dominant role of ferritin1 in iron homeostasis. In response to low iron, ferritin1 subunits and the ferritin1 oligomer abundance are increased, consistent with the increase of the corresponding mRNA. However, the iron content of the 24-mers ferritin1 protein is decreased. This suggests that increased *FER1* expression could increase capacity for iron-binding in the chloroplast of iron-limited cells, supporting a role for ferritin1 as an iron buffer. It has been established that iron deficiency promotes PSI degradation in *C. reinhardtii*. Indeed, it was observed that induction of ferritin synthesis correlated with the degree of PSI degradation during iron deficiency, and that the PSI level can be restored to normal within 24 h after iron repletion at the expense of the accumulated ferritin [66]. It indicates that the iron-ferritin is likely to participate in the fast adjustment of the photosynthetic apparatus with respect to iron availability. RNAi strains with reduced ferritin amount exhibit a delay in the degradation of PSI under iron deficiency, and these *Chlamydomonas* strains are more sensitive to photo-oxidative stress under high light conditions. Consistent with this increase in stress suceptibility, it was observed that cell growth was inhibited at high light correlatively to an increase in iron concentration in the culture medium, and that under these conditions the FER1 mRNA abundance was decreased [67]. Furthermore, the time course of the response to high light compared to the response to pro-oxidants such as Rose Bengal or H_2O_2 treatments suggests that both singlet oxygen and H_2O_2 could be involved in the high light response.

In plants, ferritin gene expression is modulated by many environmental factors including drought, cold, light intensity, and pathogen attack. Throughout their life cycle, plants often experience these stresses, which are variable in intensity, location, and duration, and which can transiently raise intracellular free iron pools, leading to an increased reactivity with oxygen to generate ROS. It is therefore very likely that plant ferritins, by buffering iron, help plants to cope with adverse situations, the deleterious effects of which would be amplified if no system had evolved to take care of free reactive iron. This function of plant ferritin is reminiscent of one of the bacterioferritin role (defence against oxidative stress rather than iron storage for metabolic purpose). However, in bacteria there are several maxi ferritin equipments and depending on the bacterial species, ferritins can have differential roles. In some cases, Ftn like ferritins are also involved in long-term iron storage. This could also happen in some plant species under conditions that remain to be defined.

5. Conclusion

Iron is a limiting factor for plant productivity and biomass production. Heme and iron–sulfur proteins play a key role in these aspects and our knowledge of the biosynthesis of these co-factors has

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recently made huge progress. However, the beneficial effect of iron on plant biomass and seed production appears dependent on the presence of ferritins. Indeed, growing a ferritin-less mutant under elevated iron conditions revealed major developmental defects associated with iron homeostasis perturbations and oxidative stress [11]. In nature, ferritin gene expression is modulated by many environmental factors. Throughout their life cycle, plants often experience these stresses, which are variable in intensity, location, duration, and which can transiently raise intracellular free iron pools, leading to an increased reactivity with oxygen. Thus, the regulation of ferritin gene expression is indicative of the importance of these proteins for the adaptive response of plants to environmental changes. The new challenge we are facing now will be to understand how these activities are integrated at the whole plant level, and what are the signaling networks and regulatory molecules responsible for the control of iron dynamics in plants.

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