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REVIEW

New insights into ferritin synthesis and function highlight a link between iron homeostasis and oxidative stress in plants

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• *Background* Iron is an essential element 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 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 differs. 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.

• *Scope* In this review, an overview of our knowledge of bacterial and mammalian ferritin synthesis and functions is presented. Then the following will be reviewed: (*a*) the specific features of plant ferritins; (*b*) the regulation of their synthesis during development and in response to various environmental cues; and (*c*) their function in plant physiology, with special emphasis on the role that both bacterial and plant ferritins play during plant–bacteria interactions. Arabidopsis ferritins are encoded by a small nuclear gene family of four members which are differentially expressed. Recent results obtained by using this model plant enabled progress to be made in our understanding of the regulation of the synthesis and the *in planta* function of these various ferritins.

• *Conclusions* Studies on plant ferritin functions and regulation of their synthesis revealed 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.

Key words: Iron, bacterioferritins, ferritins, oxidative stress, iron storage, seeds, pathogens, nutrition.

INTRODUCTION

Metal interactions with organic compounds are numerous in all living organisms. They are essential for many biochemical processes occurring within cells, and concern not only metabolism, but also some regulatory mechanisms of gene expression.

Among metals, iron is of special interest because it is required in most of the cellular redox reactions and it is one of the major metals involved in electron transfer chains. However, its strong reactivity with oxygen makes it a difficult element to handle by aerobic organisms. Indeed, both its insolubility in the form of ferric hydroxides, or its toxicity through the Fenton reaction producing hydroxyl radicals (which are among the most chemically reactive species), have introduced evolutionary constraints in order to enable this metal to be safely utilized by living organisms. The narrow efficient iron concentration required for cellular needs is strictly controlled by biological processes acting both at the transport and the storage levels. In multicellular organisms, transport mechanisms regulate iron traffic from uptake to long distance tissular distribution, and ultimately to subcellular allocation. During

developmental processes or in response to environmental challenges, buffering and transitory storage of iron also participates to maintain iron homeostasis. A specific class of iron storage proteins is implicated in this latter function: the ferritins. The main purpose of this paper is to review our current knowledge of plant ferritins, after having summarized first our current knowledge of prokaryote and animal ferritins.

GENERAL FEATURES OF FERRITINS: AN OVERVIEW

Ferritins are a broad superfamily of iron storage proteins, found in all the living kingdom, except in yeast (Andrews *et al.*, 2003; Briat *et al.*, 2006; Arosio *et al.*, 2008). Three subclasses of these proteins can be defined: (1) haem-free ferritins present both in pro- and eukaryotes; (2) haem-containing bacterioferritins, found only in bacteria; and (3) *D*NA binding *p*roteins from *s*tarved cells (Dps), called miniferritins, present only in prokaryotes (Smith, 2004). Ferritins and bacterioferritins are composed of 24 subunits whereas only 12 identical subunits form the Dps proteins. These subunits assemble in a spherical protein shell defining a central cavity able to accomodate between 2000 and 4000 ferric iron atoms for ferritins and 500 atoms for miniferritins (Andrews *et al.*, 2003; Carrondo, 2003). The oxidation of ferrous iron prior to

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ferric iron storage within the cavity is catalysed by the ferritin protein shells which possess a binuclear di-iron centre defined as the the ferroxidase centre involved in the oxidation of Fe(II) prior to its accomodation in the Fe(III) mineral core (Arosio *et al.*, 2008). Oxygen and hydrogen peroxide are the major cellular oxidants consumed during this oxidation reaction (Bou-Abdallah *et al.*, 2002; Arosio *et al.*, 2008). Sequestered ferric iron is bio-available in case of cellular needs and is non-reactive with oxygen. Besides their role in iron storage, ferritins and miniferritins are therefore also involved in protection against oxidative stress through their potential detoxification properties of excess iron, dioxygen and, to some extent, hydrogen peroxide (Zhao *et al.*, 2002; Theil *et al.*, 2006; Arosio *et al.*, 2008).

Prokaryote ferritins

Prokaryotes have three sub-classes of proteins able to store up to a few thousands of iron atoms inside a central cavity defined by the protein shell: the miniferritins (Dps), the bacterioferritins (BFR) and the bacterial ferritins (FTN).

Miniferritins, or Dps, are dodecameric ferritins which have been documented in many different bacteria species (Smith, 2004), although most of the knowledge concerning these miniferritins was obtained with *Escherichia coli*. *Escherichia coli* Dps belongs to the family of non-specific DNA-binding proteins induced in response to carbon starvation or to H_2O_2-generated oxidative stress. During exponential growth Dps is degraded by Clp proteases, whereas it is stabilized and its synthesis increased in response to stress (Stephani *et al.*, 2003). Dps participates to protect DNA from various nucleases and oxidative agents by binding to it in a non-specific manner, and consequently it triggers a low level of gene expression. The Dps protein of *E. coli* is able to oxidize Fe(II) through specific sites containing iron-binding amino acids such as aspartate, glutamate and histidine (Ilari *et al.*, 2000, 2002). It has been characterized as a dodecameric sphere of 205 kDa accommodating 500 Fe(III) atoms in its central cavity.

Bacterioferritin, encoded by the *BFR* gene in *E. coli*, consists of 24 identical polypeptides of 18·5 kDa assembled in a hollow sphere of 452 kDa. It is able to accomodate about 1800 Fe(III) atoms in its central cavity. The major peculiarity of bacterioferritins is to contain one haem molecule for two subunits; i.e. 12 haem residues per bacterioferritin molecule–, a methionine residue of each subunit serving as haem ligands (Cheesman *et al.*, 1990, 1993). Although the function of these haem residues is not well characterized, engineered haem-free BFR load about four times more iron *in vivo* than wild-type BFR (Andrews *et al.*, 1995).

Because BFR, as Dps, preferentially use H_2O_2 rather than O_2 during the Fe(II) oxidation process, they are also considered to play a major role in protection against oxidative stress.

Bacterial ferritin has been largely documented in *E. coli*. It is a non-haem 24-mers protein of 465 kDa, each 19·5-kDa subunit being encoded by the *FTN-A* gene, and having a sequence more related to the human H ferritin subunit than to the BFR haem-containing bacterioferritin (Izuhara *et al.*, 1991). It is able to accomodate about 2500 iron atoms in its

central cavity through a ferroxidase activity characteristic of H-type ferritins (see the section entitled Mammalian ferritins). As for BFR and Dps, the role of FTN in protecting cells against oxidative damage has been documented (Touati *et al.*, 1995; Touati, 2000). However, such an effect occurs only in a genetic background lacking the Fur transcriptional regulator of bacterial iron homeostasis (Abdul-Tehrani *et al.*, 1999).

Prokaryote maxi-ferritins (FTN and BFR) and mini-ferritins (Dps) have their expression regulated in response to iron or oxidative stress. FTN and BFR gene expression is principally regulated by the Fur–Fe^{2+} complex (Andrews, 1998; Cartron *et al.*, 2006). In addition, and to a lesser extent than some other genes, BFR mRNA abundance is regulated by the regulatory RNA RyhB. RyhB is a noncoding RNA regulated by the Fur repressor causing the rapid degradation of a number of mRNAs that encode proteins that utilize iron (Masse *et al.*, 2005). Among these proteins, RyhB regulates negatively the synthesis of Fur, therefore creating a negative feedback loop essential for the tight control of iron homeostasis within the cell and impacting indirectly the synthesis of ferritin (Vecerek *et al.*, 2007). Dps proteins are particularly important during oxidative stress or, when protein turnover releases iron (Moore *et al.*, 2005; Reindel *et al.*, 2006). In *E. coli*, *Dps* gene expression in response to hydrogen peroxide is coupled to *FTN* expression during the exponential phase via the transcriptional regulator OxyR, and to *BFR* expression during the stationary phase via both Sigma S- and the histone-like protein IHF (Altuvia *et al.*, 1994).

Many *Bacillus* spp., have the peculiarity to possess two mini-ferritin (Dps) genes in their genome but no maxi-ferritin gene. In these bacteria, H_2O_2-dependent induction of the *Dps2* gene expression occurs through modification of the PerR transcription factor by an Fe^{2+}/H_2O_2 catalysed reaction (Lee and Helmann, 2006). The *Dps1* gene is induced by non-specific oxidative stress that is Sigma B-dependent during exponential growth and Sigma B-independent during transitional growth (Antelmann *et al.*, 1997).

Mammalian ferritins

The structure–function relationships of mammalian ferritins have been widely studied (Arosio *et al.*, 2008). Mammalian apoferritins are composed of 24-heteromers, and have a molecular mass of approx. 450 kDa. The central cavity of this hollow sphere can accomodate up to 4500 iron atoms in a safe and bioavailable form, chemically defined as ferrihydrite. Subunits of about 20 kDa each are of two types referred to as H- and L-chains. H- and L-chains share approx. 55% amino acid homology. They are quantitatively differentially abundant in various tissues and, consequently, mammalian apoferritins can be quite variable in their composition depending on the number of L- and H-chains in the protein complex. For example, the heart ferritins are mainly composed of H-chains whereas the liver ferritins contain essentially L-chains. The major difference between the L- and H-chains is the presence in each H-chain of a ferroxidase centre of seven amino acid residues (Glu27, Tyr34, Glu61, Glu62, His65, Glu107 and Glu141in the human ferritin H-chain). The ferroxidase centre enables a rapid oxidation of Fe(II)

which binds to it prior to being oxidized into Fe(III), and then incorporated into the mineral core (Levi *et al.*, 1994; Le Brun *et al.*, 1995; Keech *et al.*, 1997). The L-chain has no ferroxidase activity and L-rich ferritins are therefore less efficient in rapid uptake of iron, comparatively to H-rich ferritins. However, long-term storage of iron is privileged in L-rich ferritins since L subunit specific amino acids facing the central cavity of the protein are known to facilitate the mineralization of ferric ions inside this cavity.

In vivo iron loading of ferritin may be more complex than *in vitro*. Indeed it has recently received support by the finding of a human iron chaperone able to bind ferritin and to facilitate iron incorporation both *in vivo* and *in vitro* (Shi *et al.*, 2008). This chaperone corresponds to the poly (rC)-binding protein 1 (PCBP1) which is an ubiquitous mammalian RNA-binding protein found both in the cytosol and nucleus. PCBP1 can bind with high affinity up to three Fe atoms per molecule and is found associated with ferritin only in the presence of ferrous iron.

Iron release from ferritin has been studied *in vitro*, showing that this metal is stable within the core, and can be released only slowly by strong Fe(III) chelators (Chasteen, 1998). Reducing agents, however, are well known for their ability to promote iron release from ferritin mineral cores (Cassanelli and Moulis, 2001), even without penetrating inside the cavity (Theil *et al.*, 2006). This involves the transfer of electrons through the protein shell to the cavity (Johnson *et al.*, 1999). The hydrophilic channels on the 3-fold axes of the molecule are required for iron release. The melting of the structure around these channels, caused by chaotropic agents or by the specific binding of some peptides that control the pore opening (Liu *et al.*, 2007), increase strongly the reductive release of iron *in vitro* (Jin *et al.*, 2001). The mechanism of ferritin iron release *in vivo* is much more difficult to apprehend and it is currently admitted that ferritin iron is mainly released after proteolytic degradation of the protein through different mechanisms (Truty *et al.*, 2001; De Domenico *et al.*, 2006; Kidane *et al.*, 2006). One of them is likely to involve ubiquitination and the proteasome pathway. This proteasome-dependent pathway for ferritin degradation and iron release could be enhanced by oxidative damage (Mehlhase *et al.*, 2005). So far it is still unclear if the reductive processes necessary for ferritin iron release produce pro-oxidant intermediates which are potentially toxic.

Concerning their subcellular localization, although animal ferritins are mostly found within the cytoplasm as soluble proteins, there are few reports of the presence of ferritins inside the nucleus, where they could protect DNA against oxidative stress, or even regulate the transcription of some genes (for a review, see Arosio *et al.*, 2008). Ferritins have also been reported to be located in the mitochondria of some cell types, where they are encoded by an intronless gene (for a review, see Arosio *et al.*, 2008). Animal ferritins are not only intracellular proteins since they can be secreted when synthesized as precursors in worm or insects cells, or even as mature polypeptides in certain mammalian cell types (for a review, see Arosio *et al.*, 2008). The tissue-specific H : L ratio of ferritin subunits is determined by the transcriptional regulation of ferritin genes during development. Mammalian ferritin expression is also regulated at the transcriptional

level in response to inducers promoting the antioxidant response. It requires antioxidant-responsive *cis* elements (AREs) found in the upstream promoter region of ferritin genes, and able to recruit Bach1 and maf proteins as transcription factors (Torti and Torti, 2002; Hintze and Theil, 2005; Theil, 2007). Although of importance, these transcriptional regulations received less attention than the translational control of ferritin mRNA expression in response to iron through the iron responsive element (IRE)/iron response protein (IRP) translational repression system (for a review, see Theil, 2007; Arosio *et al.*, 2008). IREs are specific hairpin structures found in the 5′ UTR of ferritin mRNA which are able to bind with high affinity the repressors IRP1 and IRP2. The IRE-binding activity of the IRPs is regulated by the cellular iron and redox status. Under low iron conditions, the IRE/IRP complex prevents ribosome scanning and translation of ferritin transcripts. Under high iron conditions, IRP1 contains an Fe/S cluster, exhibits aconitase activity and looses its RNA-binding activity. The transition between IRP1 and cytosolic aconitase activity is affected by protein phosphorylation and iron-induced proteasomal degradation (Fillebeen *et al.*, 2003; Clarke *et al.*, 2006; Wang *et al.*, 2007). In contrast to IRP1, IRP2 is not an aconitase. This translational repressor is degraded under high iron conditions via haem interaction, and its *S*-nitrosylation has been suggested to regulate its stability via the ubiquitin proteasome pathway (Kim and Ponka, 2002; Kim *et al.*, 2004). IRP2 appears to be the major ferritin translational regulator *in vivo* (Meyron-Holtz *et al.*, 2004; Galy *et al.*, 2005), and the L-ferritin is more tightly regulated by iron at a post-transcriptional level than H-ferritin (Sammarco *et al.*, 2008).

DISTINCTIVE FEATURES OF PLANT FERRITINS

Plant ferritins have a eukaryotic origin, and their amino-acid sequence is well conserved with animal ferritin (Ragland *et al.*, 1990; Andrews *et al.*, 1992). A specific feature, however, has to be noticed at this level. Plant ferritins are synthesized as precursors with a plant-specific N-terminal amino acid extension. This extension is composed of two parts. The shorter one, named extension peptide (EP) belongs to the mature ferritin subunit and is involved in the control of protein stability during iron exchange (Van Wuytswinkel and Briat, 1995; Van Wuytswinkel *et al.*, 1995). Upstream of the EP, a cleavable transit peptide sequence responsible for subcellular targeting of ferritin subunits is observed (Ragland *et al.*, 1990; Proudhon *et al.*, 1989). Indeed, plant ferritins have never been reported to be localized in the cytoplasm. Until recently, they were described as exclusively targeted to non-green plastids, being found accumulated in chloroplasts only in response to stresses (Fobis-Loisy *et al.*, 1995; Briat *et al.*, 1995). In 2004, it was reported that plant ferritins can also be observed in mitochondria, sharing therefore this cellular localization with some animal ferritins (Zancani *et al.*, 2004). However, in contrast to animal ferritins, the gene encoding plant mito-ferritin is not different than others, and the product it encodes could be dual targeted to both mitochondria and plastids. The function of ferritin within plant mitochondria is so far not documented.

As a result of its presence in plastids, a prokaryotic-type environment, the structure of plant ferritin mineral cores is amorphous because of its high P : Fe ratio of 1 : 3, which is different from the ferrihydrite cristal structure found within animal ferritin (Wade *et al.*, 1993; Waldo *et al.*, 1995). The environment of the protein (i.e. richness in P) is more responsible for this difference than the structure of the protein itself since *in vitro*, in absence of P, plant apoferritin can load iron within its central cavity in the form of pure ferrihydrite (Wade *et al.*, 1993; Waldo *et al.*, 1995).

In addition to their specific NH_2-terminal extension plant ferritin subunits have some additional features compared with their animal counterpart. Although the predicted 3-D structure of the pea seed ferritin can be superimposed ± 1 Å with mammalian H ferritin (Lobréaux *et al.*, 1992*a*), there is no distinguishable H- or L-subunits in the plant. Indeed, all the subunits characterized so far contain both a typical H-type ferroxidase centre, and all the amino-acid residues characteristics of an L-type subunit for a better iron nucleation within the cavity (Lobréaux *et al.*, 1992*a*). Plant ferritin can therefore be considered as an H/L hybrid ferritin. In this respect, it is interesting to notice that *in vitro*, the ferroxidase activity of purified ferritin is intermediate between recombinant H- or L-human ferritin (Van Wuytswinkel and Briat, 1995).

All eukaryotic ferritins when assembled as 24-mer exhibit channels in the 3-fold and 4-fold symmetry axes of their three dimensional structure. In plant ferritins, both these channels are hydrophilic whereas animal ferritins have their 3-fold channels built up with hydrophilic residues and their 4-fold channels with hydophobic residues. The significance of this difference, if any, is unknown (Lobréaux *et al.*, 1992*a*).

Finally, a major difference between plant and other eukaryotic ferritins concerns the regulation of their synthesis in response to excess of iron (see below). Whereas this regulation occurs mainly at the translational level through the IRE/IRP system in animals (see the section above; for a review, see Theil, 2007; Arosio *et al.*, 2008), the regulation of plant ferritin gene expression in response to this metal is achieved through a transcriptional control (Lescure *et al.*, 1991). The involvement of an aconitase/IRP switch in the regulation of iron homeostasis in plants has been suggested (Peyret *et al.*, 1995; Navarre *et al.*, 2000). However, no IRE can be predicted within the 5′ UTR of plant ferritin mRNAs, and the iron-mediated expression of the *AtFer1* and *AtFer3* ferritin genes is unaffected in *Arabidopsis thaliana* aconitase null mutants (Arnaud *et al.*, 2007). Therefore, the existence of an aconitase/IRP switch in plants to regulate ferritin gene expression is very unlikely to occur.

PLANT FERRITIN SYNTHESIS

Important data documenting the regulation of ferritin synthesis have been obtained with maize (Lobréaux *et al.*, 1992*b*, 1993; Savino *et al.*, 1997; Petit *et al.*, 2001*b*), pea (Lobréaux and Briat, 1991) and soybean (Lescure *et al.*, 1991; Wei and Theil, 2000). However, Arabidopsis is now offering the best opportunity to characterize exhaustively the differential expression of the four members of the ferritin gene family present in the genome of this model plant, both during the course of development and in response to environmental signals (Petit *et al.*, 2001*a*; Ravet *et al.*, 2009).

Developmental regulation of ferritin synthesis

The developmental regulation of ferritins is known to occur from electron microscopy studies in which their presence in various organs and tissues has been reported (for a review, see Seckback, 1982). Ferritin naturally occurs in plant tissues such as cotyledons, root and shoot apices, cells of vessels, in vascular cambium, reproductive cells and senescing cells. However, it is only recently that developmental regulation of plant ferritin has been documented at the molecular level, at defined developmental stages of leaves (Theil and Hase, 1993) and nodules (Ragland and Theil, 1993; Kimata and Theil, 1994), as well as during the life cycle of the pea (Lobréaux and Briat, 1991) and *A. thaliana* (Petit *et al.*, 2001*a*) plants.

In pea and *A. thaliana*, ferritin accumulates in the cotyledons and embryo axis of seeds, while the amount of ferritin in vegetative organs is low (Lobréaux and Briat, 1991; Petit *et al.*, 2001*a*). Post-transcriptional control at the protein stability level participates to regulate the amount of seed ferritin. Disappearence of ferritin protein from pea seed embryo axes during the first days following germination is accompanied by the appearance of polypeptides of lower molecular weight than the basic ferritin subunit (Lobréaux and Briat, 1991). A mechanism responsible for this specific processing has been proposed, based on iron release experiments performed *in vitro* (Laulhère *et al.*, 1989, 1990). *In vitro* release of ferritin iron, when reduced by ascorbate or light, induces conversion of the ferritin subunit from 28 kDa to 26·5 and 25 kDa. As a consequence, pea seed ferritin then has a tendency to aggregate and to become insoluble (Laulhère *et al.*, 1989). Fenton chemistry has been involved in this process since protein cleavage is inhibited by free radical scavengers as well as by iron chelators (Laulhère *et al.*, 1989, 1990). These free radical cleavages occur within the plant-specific NH_2-terminus of the pea seed ferritin subunit (Laulhère *et al.*, 1989). These observations have been confirmed by the demonstration that recombinant pea seed ferritin, depleted of its NH_2-terminus, is less soluble than recombinant wild-type pea seed ferritin (Van Wuytswinkel *et al.*, 1995). A model explaining the mechanism of ferritin degradation during germination has been proposed (Lobréaux and Briat, 1991). Alternatively, it has been proposed recently that the 28-kDa and 26·5-kDa ferritin subunits from pea seed could be encoded by two different genes, instead of having the smaller subunit produced by processing of the 28-kDa isoform (Li *et al.*, 2009)

Another example of developmental control of ferritin synthesis has been reported in leaves. Indeed the first evidence of such a regulation was suggested because the concentration of ferritin mRNA measured in mature soybean leaves was found to be higher than in young leaves (Ragland *et al.*, 1990). Using the determinate nature of leaf development in maize, a high level of ferritin is only detected in the youngest section of the leaf whereas in the middle of this organ the ferritin concentration reaches its minimum (Theil and Hase, 1993). An unco-ordinated change in ferritin mRNA and ferritin protein concentrations was also reported between the

various developmental stages of this leaf development, strengthening the hypothesis that a post-transcriptional control is likely to be an important level of regulation of ferritin synthesis during plant development (Theil and Hase, 1993).

In legumes, iron is also important for nitrogen fixation within the nodules where iron-proteins such as leghaemoglobin and nitrogenase play a key role. Variations in ferritin abundance in soybean nodules depending on their age, on interactions between the *Bradyrhizobium* strain and the host cultivar, and on mutations affecting the bacterial strain have been reported (Ko *et al.*, 1987). In effective *Bradyrhizobium/* soybean symbiosis, ferritin has been observed only in young nodules. By contrast, in ineffective nodules produced by *Bradyrhhizobium* mutants, ferritin is found in nodules of all ages. Hence, ferritin is present in nodules when they are not functional. During the course of soybean nodule ageing, ferritin mRNA concentration remains elevated while the protein concentration decreases 4- to 5-fold (Ragland and Theil, 1993). It again indicates the occurence of a post-transcriptional control of ferritin abundance during a plant-specific developmental programme. During this process of soybean nodule ageing, before complete disappearance of the ferritin protein, breakdown products have been observed (Ragland and Theil, 1993), reminiscent of those observed during ferritin processing following seed germination (Lobréaux and Briat, 1991). Whether reactive oxygen species (ROS)-mediated signalling of ferritin degradation within ageing nodules occurs, as suggested for germinating seeds (Laulhère *et al.*, 1989; Lobréaux and Briat, 1991), remains to be addressed.

In *A. thaliana*, ferritins are encoded by a small gene family of four members. The analysis of the tissue-specific expression of each of these genes at the transcript level, and kinetically during the life cycle of a plant was therefore straightforward to perform (Petit *et al.*, 2001a; Fig. 1). Both AtFer1 and AtFer3 mRNA transcripts are present in rosette leaves, their abundance being higher at later developmental stages. In roots, AtFer1 mRNA has the same abundance at three various time points of the life cycle, whereas AtFer3 mRNA seems to be less abundant than AtFer1. In floral stalks, AtFer1 and AtFer3 transcripts are found in the stem and the leaves, Atfer1 transcript being abundant in this organ compared with AtFer3 which is weakly detected, with no variation between the two time points analysed. Both AtFer1 and AtFer3 mRNA abundances are important in flowers and

these transcripts are still detected in siliques. In contrast, they are not observed either in dry siliques or in dry seeds. Both AtFer1 and AtFer3 mRNA are present in tissues during the early stages of germination, after 20 h or 30 h of seed imbibition and in 5-d-old germinating plantlets. The expression pattern of AtFer2 mRNA is markedly different from those observed for AtFer1 and AtFer3 mRNAs since (*a*) it is abundantly present in mature siliques and dry seeds where no other AtFer transcripts are detected, and (*b*) conversely it is almost undetectable in roots, rosette or floral stalk leaves, stem, flowers, immature siliques or germinating seeds. The observation that AtFer2 expression is restricted to the seed is consistent with the observation that this gene is activated in response to exogenous ABA application, as was also reported for its ZmFer2 maize orthologue (Lobréaux *et al.*, 1993; Fig. 2). The expression pattern of AtFer4 diverges importantly from what is described above for AtFer1, AtFer3 and AtFer2. Its expression is restricted to the floral stalk and in flowers, with a maximum after pollination.

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 has recently been achieved (Ravet *et al.*, 2009). It makes it possible to conclude that only AtFer1 exists mainly 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 AtFer3 and AtFer4 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 (Petit *et al.*, 2001a). It is therefore interesting to notice that Arabidopsis ferritin subunits can be observed, as in pea (Laulhere *et al.*, 1989; Lobreaux *et al.*, 1991) and in soybean (Masuda *et al.*, 2001) as two polypeptides of different molecular weight. 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 transport of ferritin subunit precursor into the chloroplast. The second domain is part of the mature protein, and named extension peptide. As indicated above its function could be related to protein stability, and it could be cleaved during the germination process of pea seeds through free radical damages (Lobreaux *et al.*, 1991; Van Wuytswinkel
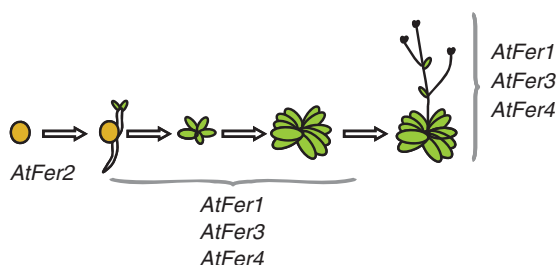

Fig. 1. Tissue-specific expression and developmental regulation of ferritin synthesis in Arabidopsis. The expression of the *AtFer2* ferritin gene is restricted to mature seeds. In vegetative and reproductive organs, from the germination stage to flowering, *AtFer-1*, *-3* and *-4* genes are kinetically and differentially expressed in various tissues and organs (Petit *et al.*, 2001a; Ravet *et al.*, 2009)
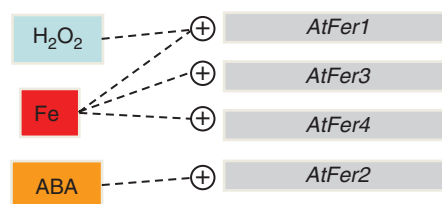

Fig. 2. Differential expression of Arabidopsis ferritin genes in response to exogenous signals. Iron excess treatment (Fe) leads to *AtFer-1*, *-3* and *-4* gene expression, whereas H_2O_2 application impacts positively only on the expression of the *AtFer1* gene. Consistent with its seed-specific expression, the *AtFer2* gene is the only Arabidopsis ferritin gene to be expressed in response to application of exogenous abscisic acid (ABA; Petit *et al.*, 2001a).

et al., 1995). Another mechanism of ferritin-subunit processing leading to a 26·5-kDa subunit from a mature 28-kDa-subunit has been proposed. Sequencing of purified assembled ferritin from soybean seeds enabled two subunits, H-1 and H-2, to be identified (Masuda *et al.*, 2001). H-2 is an unprocessed 28-kDa subunit, whereas H-1 exhibited a 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). Interestingly, in *A. thaliana* only the FER1 subunit exists in a 28-kDa form, whereas subunits encoded by *AtFer2*, *AtFer3* and *AtFer4* genes are only present as a 26·5-kDa polypeptide (Ravet *et al.*, 2009). 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 the observation obtained with soybean ferritin, and indicating that a C-terminal cleavage could occur at a conserved arginine residue.

Ferritin synthesis in response to iron excess

In maize, the *ZmFer1* ferritin gene is activated in response to iron excess (Fobis-Loisy *et al.*, 1995), and its promoter was used to search for iron-dependent *cis*-regulatory elements (Petit *et al.*, 2001*b*). The Fe-dependent up-regulation of *ZmFer1* expression can be antagonized by antioxidants and PP2A-type phosphatase inhibitors (Savino *et al.*, 1997). The 'Fe excess' signal leading to the expression of the *ZmFer1* gene could therefore require oxidative stress and phosphorylation/dephosphorylation events to be transduced. Serial deletions and site-directed mutagenesis enabled a 15-bp sequence named IDRS (iron-dependent regulatory sequence) to be characterized within the proximal part of the promoter of the *ZmFer1* ferritin gene, necessary for the repression of this gene under Fe-deficiency conditions (Petit *et al.*, 2001*b*). In Arabidopsis, the *AtFer1* gene is the orthologue of the maize *ZmFer1* gene, its expression in response to Fe excess also being antagonized by antioxidants and Ser-Thr phosphatase inhibitors (Gaymard *et al.*, 1996; Petit *et al.*, 2001*b*). Sequence alignments show that the IDRS sequence defined in maize is highly conserved in Arabidopsis. Further analysis

has shown that this sequence is functional *in planta* and, as demonstrated in maize, involved in the transcriptional repression of *AtFer1* expression under iron-deficient conditions (Petit *et al.*, 2001*b*). The IDRS sequence is also required for dark-induced senescence activation of *AtFer1* expression but not for age-dependent senescence induction (Tarantino *et al.*, 2003). *AtFer1* promoter activity in leaves is observed mainly in the vicinity of the vessels and mutations within the IDRS do not alter this localization. In roots, the *AtFer1* promoter activity was restricted to the endoderm. However, IDRS mutagenesis resulted in an extension of the promoter activity to the root cortex and epidermis (Tarantino *et al.*, 2003). These results indicate that, under standard Fe nutrition conditions, the IDRS could be involved in the repression of expression of the *AtFer1* gene in the cortex and epidermis cells. In the absence of a functional IDRS, *AtFer1* gene repression would not occur any more, leading to an extension of the root expression territories of this gene.

By combining pharmacological and imaging approaches in an Arabidopsis cell culture system, several elements of the signal transduction pathway leading to the increase in AtFer1 transcript level after iron treatment have been clarified (Arnaud *et al.*, 2006). Nitric oxide (NO) quickly accumulates in plastids after excess-iron treatment. This compound acts downstream of iron and upstream of a PP2A-type phosphatase, consistently with a previous report having shown that NO is involved in the transduction pathway between the 'Fe excess' signal and *AtFer1* promoter de-repression, in an IDRS-, Ser-Thr phosphatase-dependent manner (Murgia *et al.*, 2002). Furthermore, the repressor required for the repression of the *AtFer1* gene transcription under low iron conditions is unlikely to be a transcription factor directly bound to the IDRS. It is rather a protein acting upstream in the pathway, which could be ubiquitinated upon iron treatment and subsequently degraded through a 26S proteasome-dependent pathway (Arnaud *et al.*, 2006; Fig. 4).

Kinetics of Arabidopsis *AtFer3* transcript accumulation in response to Fe overload are very similar to one of the *AtFer1* genes, both in roots and shoots. In contrast, the *AtFer2* gene is not induced by Fe excess and *AtFer4* mRNA abundance increases only in leaves with different kinetics than *AtFer1* and *3* transcripts (Petit *et al.*, 2001*a*).

FIG. 3. Sequence alignment of the C-terminal parts of soybean and Arabidopsis ferritin subunits. Identical and similar residues between at least three proteins are boxed in black and grey, respectively. Amino acids are numbered from the translational start methionine. The arginine in position 234 of Soy H-1 subunit was shown to be the last residue of the processed 26·5-kDa form (Masuda *et al.*, 2001). This residue and the corresponding arginines in Arabidopsis subunits are indicated in red.
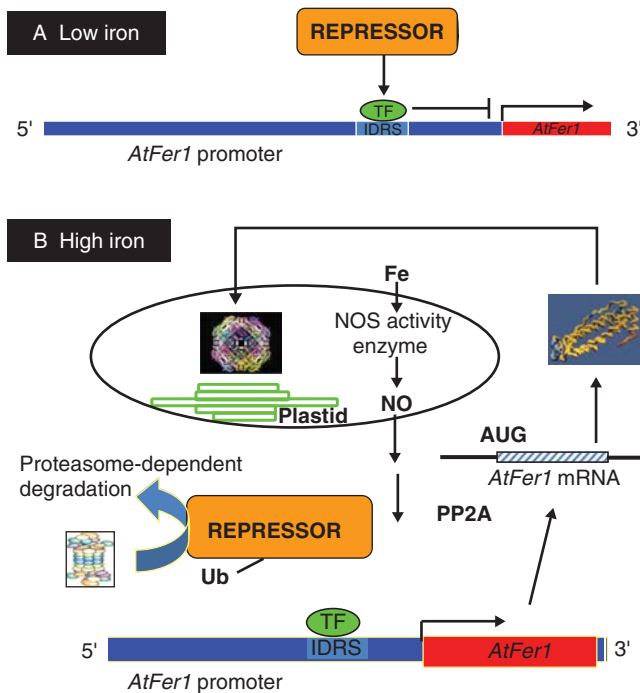
FIG. 4. A working model to explain the control of the *AtFer1* gene expression in response to iron. (A) Under low-iron conditions, a repressor not directly bound to the *AtFer1* promoter would interact with the transcription factor which recognizes the iron-dependent regulatory sequence (IDRS), leading to the repression of *AtFer1* gene expression. (B) Under high-iron conditions, an enzymatically produced nitric oxide (NO) burst occurs within the plastids, preceding ubiquitination and proteasome-dependent degradation of the repressor. De-phosphorylation events depending upon a PP2A phosphatase activity would also occur. These events lead to a de-repression of the *AtFer1* gene expression (Arnaud *et al.*, 2006). The corresponding ferritin transcript is then translated to give the ferritin precursor polypeptide which is transported to plastids where it is assembled in the 24-mers ferritin protein.

Comparison of the promoter sequence of the four Arabidopsis ferritin genes revealed the presence of an IDRS-like element in the same promoter region of the *AtFer2*, *-3* and *-4* ferritin genes as the functional IDRS characterized in the *AtFer1* promoter. However, it remains to be determined whether or not these IDRS-like are functional. Beside the IDRS, another *cis*-regulatory element than the IDRS, named FRE (Fe responsive element), has been characterized in the promoter region of a soybean ferritin gene (Wei and Theil, 2000); no such element can be observed in any of the four Arabidopsis ferritin genes (Petit *et al.*, 2001a). No *trans*-acting factors interacting with these *cis*-regulatory sequences and involved in plant ferritin gene expression have been characterized so far.

PLANT FERRITIN FUNCTIONS: STORAGE VERSUS STRESS PROTECTION

Despite a wealth of information obtained on ferritin biochemistry or expression in various plant species, the function of this iron-storage protein in plant physiology and development remained largely unknown. Most of the hypotheses formulated regarding ferritin functions in plants were based on correlations between localization of the proteins and responses of their expression to environmental factors and at various developmental stages. Investigating the functions of plant ferritins *in planta* has remained a difficult task since higher plants possess multiple gene copies coding for these proteins (Fobis-Loisy *et al.*, 1995; Wardrop *et al.*, 1999; Masuda *et al.*, 2001, 2007; Petit *et al.*, 2001; Strozycki *et al.*, 2003; Dong *et al.*, 2007). Furthermore, the high degree of sequence identity between the members of this multigenic family suggests a possible functional redundancy between the different ferritin subunits. *Arabidopsis thaliana* with its four ferritin genes offered the opportunity to investigate the biological function of plant ferritin through the combination of reverse genetic and physiological approaches. In Arabidopsis, a knock-out mutant in the *AtFer1* gene has been isolated and characterized recently. The *fer1* knock-out mutant exhibits an increased sensitivity to *Erwinia chrysanthemi* infection (Dellagi *et al.*, 2005), and a slightly accelerated senescence (Murgia *et al.*, 2007), but no major phenotype alteration is observed, strengthening the hypothesis of a functional redundancy between the four Arabidopsis gene products. New insights into ferritin function in Arabidopsis have been provided through the recent study of multiple knock-out mutants devoid of ferritin in seeds (*fer2*) or in vegetative and reproductive organs (*fer1-3-4*). This work has clearly demonstrated that ferritins are not likely to be an essential iron source for plant development, but that they play a significant role in the defence machinery against oxidative stress (Ravet *et al.*, 2009).

Ferritin is not a major player of iron storage in Arabidopsis

During the last decades, ferritin has been considered to be the major iron storage protein in plants, in particular in seeds (Hyde *et al.*, 1963). This hypothesis has been reinforced by studies on seed formation, in particular in pea. An important amount of iron is stored in pea seeds (Lobréaux and Briat, 1991). Immunodetection experiments revealed that ferritin subunits accumulated in embryo during pea seed maturation and remained present in dry seeds (Lobréaux and Briat, 1991; Marentes and Gruzak, 1998). The amount of iron stored inside ferritins is estimated to be 92% of the total seed iron content in the pea embryo axis (Marentes and Gruzak, 1998), leading to the consideration that this protein is the major form of iron storage in seeds. During germination, ferritins are degraded, and the iron released during this process has been postulated to be the main source of iron for the developing plant. In leaves, ferritins accumulated mainly in undifferentiated plastids of young leaves and become almost undetectable in the chloroplasts of the mature leaves (Theil and Hase, 1993). Thus, ferritins were hypothesized to be potential iron donors in the build-up of a functional photosynthetic apparatus where iron is mostly present as Fe–S clusters. In most of these proteins, iron is present as Fe–S clusters. In Arabidopsis mitochondria, the requirement of the frataxin protein as the iron donor for Fe–S cluster biosynthesis has been recently demonstrated (Busi *et al.*, 2006). Since plastids are autonomous for their Fe–S cluster biosynthesis (Balk and Lobréaux, 2005), ferritins were suggested to fulfil the same function in these organelles as frataxin in mitochondria.

Recently, the importance of seed ferritins as the iron donor for Fe-proteins during the germination process has been challenged. Indeed, vacuolar iron remobilization by the NRAMP3

and NRAMP4 tonoplast metal effluxers is essential for germination to take place (Lanquar *et al.*, 2005). Moreover, *fer2* plants devoid of ferritin in seeds exhibit no obvious macroscopic phenotype; the seed iron content, the germination rate, and the proper development of the mutant plants are unaffected, even under iron-limiting conditions (Ravet *et al.*, 2009). Such observations are consistent with the calculation that iron contained in ferritins of Arabidopsis seeds represent no more than 5% of the total seed iron (Ravet *et al.*, 2009). To the best of our knowledge, seed iron ferritin content has been documented only for Arabidopsis and pea (Marentes and Gruzak, 1998; Ravet *et al.*, 2009). It would be interesting therefore in the future to have comparative results with other plants.

In view of the elevated abundance of ferritins in young leaves, it was expected that an alteration in these organs would be observed in mutant plants lacking ferritins in vegetative tissues. Surprisingly, no such alteration of young leaves occur in a *fer1-3-4* mutant plant devoid of ferritins in vegetative organs, and grown under standard conditions. Moreover, in mature leaves, photosynthesis is not significantly affected by the absence of ferritins; chlorophyll fluorescence parameters and CO_2 fixation capacity are similar in wild-type and ferritin-less leaves. It can therefore be concluded that ferritins are not necessary for proper plant development, revealing that they do not constitute a major iron source for plant development. Consistently, chloroplast functionality is not dependent on ferritin presence, suggesting that these proteins do not fulfill the same function in the plastidial Fe–S cluster biosynthesis pathway that frataxin does in mitochondria.

Ferritin: a link between iron and ROS metabolisms

The relationship between iron homeostasis and oxidative stress is clearly established in bacteria, yeast and animals, where the activity of key factors regulating iron homeostasis are modulated by oxidative stress (Rouault and Klausner, 1996; Touati, 2000; Hantke *et al.*, 2001; Kaplan *et al.*, 2006; Theil, 2007; Toledano *et al.*, 2007). In plants, such a link arises from both physiological and molecular evidence. Several studies indicate that iron excess, or mutations leading to an abnormally high level of iron, lead to an increase in ROS production and to the promotion of oxidative stress responses (Pich *et al.*, 1994; Kampfenkel *et al.*, 1995; Caro and Puntarulo, 1996; Pekker *et al.*, 2002). In addition, physiological disorders due to iron toxicity responsible for necrotic spots in leaves have been known for a long time (Ponnamperuma *et al.*, 1955). As described above, ferritin synthesis is activated at the transcriptional level by iron or H_2O_2 treatments and antagonized by antioxidant molecules. Furthermore, pro-oxidant treatments such as NO or ozone applications, as well as high light intensity, are known to induce ferritin synthesis.

Links between ferritin functions and ROS management have been deduced from the characterization of tobacco transgenic plants over-expressing ferritins. On one hand, plants over-expressing ferritin are less sensitive to methylviologen-promoted oxidative stress. However, on the other hand, ROS detoxifying enzymes are always activated in such plants even when grown under standard non-stressful conditions

(Zer *et al.*, 1994; Briat *et al.*, 1999; Deák *et al.*, 1999; Van Wuytswinkel *et al.*, 1999). These data suggest that the control of ferritin synthesis is required for a proper maintenance of the cellular redox status. A clear relationship between iron and ROS has been recently demonstrated by using Arabidopsis ferritin-less mutants (Ravet *et al.*, 2009). The *fer2* mutant plant, devoid of seed ferritins, exhibits a comparatively higher sensitivity to methylviologen treatment during germination than to the wild-type plant. Under standard growth conditions, the absence of ferritins in the *fer1-3-4* mutant plants leads also to an increased accumulation of ROS in leaves and flowers. However, this mutant can bypass the lack of safe iron storage within ferritins by increasing its ROS detoxifying enzyme activities in order to avoid cellular damages. Thus, despite the energy cost of such a compensation, the development, growth and fertility of the mutant plants lacking ferritins are similar to the those of wild-type plants, except when they are grown under high iron-concentration conditions.

Ferritin requirement for beneficial impact of high iron availability to plants

Ferritins are abundant when plants are grown under high-iron conditions. In such conditions, detoxifying enzymes are strongly induced to avoid deleterious ROS accumulation. This increase of ROS-detoxifying mechanisms in plants devoid of ferritins is not sufficient to bypass the deleterious accumulation of free iron. Consequently, pleiotropic defects both in vegetative and reproductive organs strongly impaired the growth and fertility of the ferritin-less plants (Ravet *et al.*, 2009). Thus, the beneficial effect of elevated iron concentrations in the environment measured by the increased biomass of wild-type plants, is completely abolished when plants are devoid of ferritins. The growth decrease of Arabidopsis *fer1-3-4* mutant plants has been shown to result from reduced carbon assimilation (Ravet *et al.*, 2009).

Relationships between photosynthetic activity, ROS production, iron reactivity and ferritins have been evoked. In Arabidopsis, ferritins accumulate strongly in response to photo-inhibition (Murgia *et al.*, 2001). Over-expression of ferritins in transgenic plants has led to divergent conclusions concerning their involvement in the protection against oxidative stress upon light- or cold-mediated photo-inhibition (Murgia *et al.*, 2001; Hegedus *et al.*, 2008). This link between photosynthetic apparatus activity and the protective role of ferritins has been reinforced by the study of ferritin mutants in the green algae *Chlamydomonas reinhardtii* (Busch *et al.*, 2008). In this organism, ferritins have been shown to be necessary for rapid remodelling of the photosynthetic apparatus and to minimize photo-oxidative stress in response to iron availability. In contrast, in the Arabidopsis *fer1-3-4* mutant, chlorophyll fluorescence measurements have shown that PSII efficiency is not affected. Thus, the reduced carbon assimilation observed in this mutant is not a consequence of an alteration of the electron transfer through the photosynthetic apparatus (Ravet *et al.*, 2009), but is more likely originating from a ROS-mediated alteration of Calvin cycle efficiency.

The drastic decrease in fertility of the *fer1-3-4* mutant when grown in high-iron condition has been shown to be

independent of the absence of ferritins in the rosette. The pleiotropic development defects observed in *fer1-3-4* flowers originate from a huge iron overload in this organ, accompanied by a deregulation of many genes related to iron transport and homeostasis in stems and flowers (Ravet *et al.*, 2009). This suggests that modulation of ferritin expression is essential for the plant to establish a balance between availability of iron for metabolism, and sequestration of this metal to avoid subsequent ROS-related damages.

FERRITINS AND PLANT–MICROBE INTERACTIONS

It is well established that part of the innate immunity in vertebrates occurs through withholding iron, which constitutes a powerful antimicrobial mechanism (Ong *et al.*, 2006). Competition for iron nutrition between microbial pathogens and their potential hosts is therefore central for an infection to be successful or not. In plants, the role of iron in pathogenicity has been well studied with *E. chrysanthemi* infection of saintpaulia or Arabidopsis plants, evidencing the role of high-affinity iron uptake systems mediated by the chrysobactin and achromobactin siderophores (Expert, 2005). Of particular interest is the observation that the expression of *Erwininia* genes involved in siderophores mediated iron uptake or encoding pectate lyases important for virulence are controlled by Fur, an iron-regulated transcriptional repressor (Franza *et al.*, 2002). It became therefore clear that after penetrating their host, bacteria express their virulence in relation to their iron requirements.

Of interest within the frame of this review are the two following questions. What do we know about: (1) the function of bacterial ferritins in plant–bacteria interactions; and (2) the role of plant ferritin in plant immunity?

Four loci have been identified in *E. chrysanthemi* (*fnA*, *bfr*, *dps1* and *dps2*) encoding, respectively, a ferritin, a haem-containing bacterioferritin and two miniferritins (Boughammoura *et al.*, 2007). *bfr* and *ftnA* mutants have been produced (Boughammoura *et al.*, 2008) making it possible to demonstrate that, unlike the *bfr* mutant, the *ftnA* mutant has increased sensitivity to iron deficiency and to redox stress conditions. Furthermore, FtnA and Bfr contribute differentially to the virulence *of E. chrysanthemi* depending on the host. *ftnA*, *bfr*, and *bfrftnA* mutants display a delay in the appearance of symptoms of maceration onto chicory leaves comparatively with wild-type bacteria. In contrast, using saintpaulia as host plants reveals a lag in the appearance of maceration symptoms only for the *ftnA* mutant. The *bfr* and *bfrftnA* mutants are as virulent as the wild-type strain on saintpaulia. More recently, a functional link between bacterioferritin (Bfr), bacterioferredoxin and the Suf protein machinery involved in Fe–S cluster biogenesis and assemby onto apoproteins has been shown to be important for an optimal growth of *E. chrysanthemi*, and a balanced distribution of iron between essential metalloproteins (Expert *et al.*, 2008).

Attention to the role that plant ferritins could play during plant–pathogen interactions came first from the observation made by Neema *et al.* (1993), revealing that iron incorporated into plant ferritins drastically decreases in soybean suspension cells challenged with *E. chrysanthemi*. The role of plant ferritins in defence against pathogens has been strengthened by the observation that transgenic tobacco plants accumulating alfalfa ferritin in their leaves exhibited tolerance to necrotic damage caused by viral (tobacco necrosis virus) or fungal (*Alternaria alternata*, *Botrytis cinerea*) attacks (Deák *et al.*, 1999). An increase in the expression of the *StF1* potato ferritin gene in response to infection by *Phytophthora infestans* was also reported, but in that case, the increased ferritin gene expression was not enough to confer resistance to *P. infestans* (Mata *et al.*, 2001). More recently, a cDNA encoding the AtFer1 ferritin has been pulled out from a cDNA differential screening between *A. thaliana* plants infected or not with *E. chrysanthemi*, revealing an up-regulation of this gene in response to a bacterial infection (Dellagi *et al.*, 2005). An *A. thaliana atfer1* null mutant has been used to investigate the potential role of the AtFer1 ferritin in an iron-withholding defence mechanism activated in response to bacterial infection. Indeed, spreading of the symptoms is faster and the number of systemic infections is higher in the *fer1* mutant line in comparason with the wild-type plants. This result indicates that *Atfer1* gene is involved in a basal level of resistance of *A. thaliana* to *E. chrysanthemi* (Dellagi *et al.*, 2005). The *Atfer1* gene is highly expressed 24 h after inoculation. This up-regulation does not occur after inoculation with an *E. chrysanthemi* siderophore negative mutant, suggesting a role of siderophores in the *Atfer1* gene expression in response to *E. chrysanthemi* infection. Consistent with this statement, chrysobactin application increases Atfer1 transcript abundance, an effect which is not recorded when the siderophore is loaded with iron. The chrysobactin effect is not specific because *AtFer1* up-regulation is also observed by treating plants with desferrioxamine. Neither oxidative stress nor NO are involved in the response to chrysobactin, and the *AtFer1* gene expression in response to *E. chrysanthemi* inoculation is IDRS independent, leaving the mechanism by which siderophores up-regulate *AtFer1* Arabidopsis ferritin gene expression an open question (Dellagi *et al.*, 2005).

CONCLUDING REMARKS

Iron is a limiting factor for plant productivity and biomass production (Blain *et al.*, 2007; Cassar *et al.*, 2007; Ravet *et al.*, 2009). However, this 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 (Ravet *et al.* 2009). 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 and duration, and which can transiently raise intracellular free iron pools, leading to an increased reactivity with oxygen. Thus, the fine-tuning of ferritin gene expression is indicative of the importance of these proteins for the adaptive response of plants to environmental changes. 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.

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