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Research Article

Soybean Ferritin: Isolation, Characterization, and Free Radical Generation[F]

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Abstract

The main aim of this work was to assess the multi-task role of ferritin (Ft) in the oxidative metabolism of soybean (*Glycine max*). Soybean seeds incubated for 24 h yielded 41 ± 5 μg Ft/g fresh weight. The rate of *in vitro* incorporation of iron (Fe) into Ft was tested by supplementing the reaction medium with physiological Fe chelators. The control rate, observed in the presence of 100 μM Fe, was not significantly different from the values observed in the presence of 100 μM Fe-his. However, it was significantly higher in the presence of 100 μM Fe-citrate (approximately 4.5-fold) or of 100 μM Fe-ATP (approximately 14-fold). Moreover, a substantial decrease in the Trp-dependent fluorescence of the Ft protein was determined during Fe uptake from Fe-citrate, as compared with the control. On the other hand, Ft addition to homogenates from soybean embryonic axes reduced endogenously generated ascorbyl radical, according to its capacity for Fe uptake. The data presented here suggest that Ft could be involved in the generation of free radicals, such as hydroxyl radical, by Fe-catalyzed reactions. Moreover, the scavenging of these radicals by Ft itself could then lead to protein damage. However, Ft could also prevent cellular damage by the uptake of catalytically active Fe.

Keywords: ferritin; free radical; iron; soybean.

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Introduction

In plants, Fe is required in great quantity and plays an important role in biological processes such as photosynthesis, respiration, nitrogen assimilation, and DNA and hormone synthesis (Briat 2006). Depending on the ligands to which it is coordinated, Fe can access a wide range of redox potentials and can participate in many electron transfer reactions, spanning the standard redox potential range (Galatro et al. 2007). Solubilization and long distance allocation of Fe between organs and tissues, as well as its subcellular compartmentalization and remobilization, involve various chelation and oxidation/reduction steps, transport activities and association with soluble proteins (Briat et al. 2007). Transport, sensing and storage mechanisms should be finely coordinated to avoid Fe excess and deficiency (Galatro and Puntarulo 2010). Fe storage in plants not only takes place in the apoplastic space (Briat and Lobréaux 1997) and the vacuole (Briat and Lobréaux 1998), but in ferritin (Ft) as well.

Ft, a storage protein with a structure highly conserved among plants, animals and bacteria, is composed by 24 subunits assembled into a spherical shell. Up to 4 500 Fe atoms can be stored as an inorganic complex in a non-toxic and biologically available form (Harrison and Arosio 1996). Lescure et al. (1991) suggested that only one type of polypeptide chain was present in the multimer. However, according to Masuda et al. (2001), soybean Ft is composed of two subunits of 26.5 and 28 kDa designated as H-1 and H-2, respectively, which have cooperative functional roles in soybean seeds. An N-terminal sequence is observed in plant Ft, not present in mammalian Fts (Ragland et al. 1990), which contains an extension peptide (EP) located on the outer surface of the protein (Lobréaux et al. 1992), which might impart special oxidative activities to the protein (Zhao 2010). It has been reported a function in the

control of protein stability during Fe exchange for the EP *in vitro* (Laulhere et al. 1989; van Wuytswinkel and Briat 1995; van Wuytswinkel et al. 1995) and in the early steps of germination *in vivo* (Lobréaux and Briat 1991). Recent studies have shown that at high Fe loadings of Ft, the EP can act as a second ferroxidase center for Fe binding and oxidation followed by mineralization of the Fe core through a novel four step pathway in pea seed Ft (Li et al. 2009).

Ft seems to play an important role in the defense against oxidative stress in *Arabidopsis* (Ravet et al. 2009) since the control of catalytically active Fe could be essential to avoiding oxidative stress situations. Catalytic active Fe, the labile Fe pool (LIP), is a low-molecular-weight pool of weakly chelated Fe. It consists of both forms of ionic Fe (Fe^{2+} and Fe^{3+}) associated to a variety of ligands with low affinity for Fe ions that represents only a minor fraction of the total cellular Fe (3–5%) (Kruszewski 2004). These ligands could be citrate, and other organic ions, phosphate, carbohydrates and carboxylates, nucleotides and nucleosides, polipeptides and phospholipids (Kakhlon and Cabantchik 2002; Petrat et al. 2002; Kruszewski 2003). However, the actual nature of the intracellular ligands participating in LIP formation remains obscure (Galatro et al. 2007).

The hypothesis tested here is that Ft is not only an inert storage protein, but it also is a protein that actively participates in the oxidative metabolism of the cell by the generation of active radical species, such as hydroxyl radicals ($^{\bullet}OH$), and by decreasing the production of ascorbyl radicals ($A^{\bullet}$) upon Fe uptake. The main aim of this work is to assess the oxidative multi-tasking role of Ft isolated from soybean.

Results

Ft isolation and characterization

Ft was isolated from soybean seeds imbibed for 24 h in tap water, as described in the Materials and Methods section. The chromatographic elution profile from soybean Ft by gel filtration, obtained after the isolation procedure, is shown in **Figure 1A**. Soybean Ft eluted in a single peak, and Fe-rich fractions were collected for assessing protein content in the sample. Even though not only composition but also the sequence are critical to defining protein function, the amino acid composition of the sample was assessed, and compared with Ft from other plants sources, and with animal Ft. **Table 1** showed that glutamic acid and histidine (his) content, which seem to be involved in the ferroxidase activity (Arosio et al. 2009), are highly conserved in the shown Fts. Ft content after 24 h of seed imbibition was 41 ± 5 μg/g fresh weight (FW). Purified Ft showed two peptides (28 and approximately 26.5 kDa) in an sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), in agreement with Masuda et al. (2001) (**Figure 1B**, upper panel). The antibody obtained in mouse using the bands corresponding to 26.5 and 28 kDa molecular weight, was able to detect Ft subnunits from the total protein samples of soybean embryonic axis, and in total protein samples from soybean seeds as positive controls (data not shown). Subunit analyses indicated that the protein was mainly composed of 28 kDa subunits (**Figure 1B**, lower panel). However, other molecular mass subunits could appear according to the purity and stability of the preparation. Some

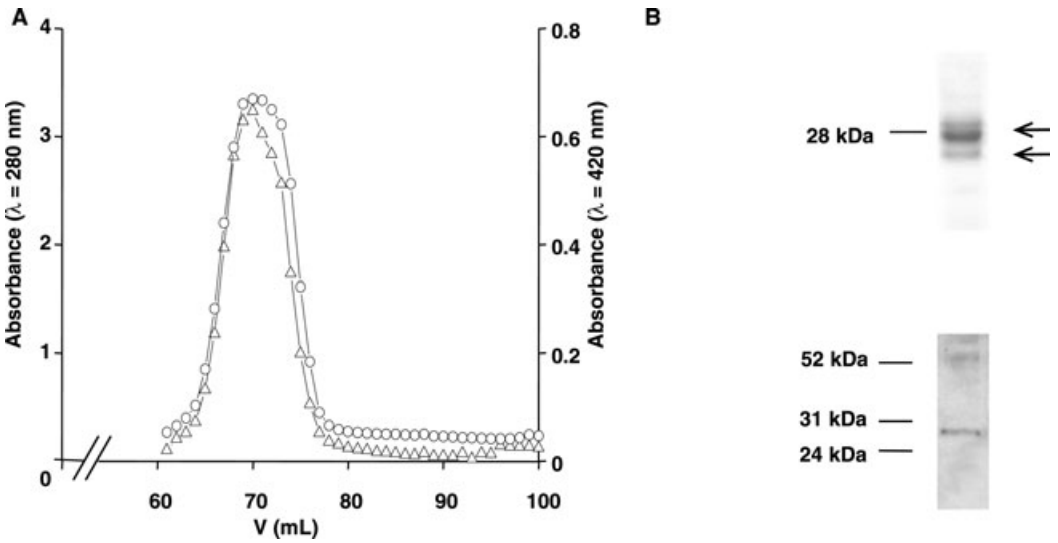

Figure 1. (A) Elution profile of extracts obtained during Ft purification through a Sephacryl S-300 column, showing the temporal correlation of the protein content (A_{280nm}) (○), and the protein bound Fe (A_{420nm}) (△). (B) Upper panel, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15%) of purified soybean Ft subunits, → indicates ~26.5 and 28 kDa bands; lower panel, immunoblotting of purified Ft from soybean seeds.

Table 1. Amino acid composition of Ft from soybean, and other different origins

Amino acid	% Residues			
	Soybean	Pea	Maize	Horse spleen
Asp	4.3	10.3	11	11.9
Thr	6.4	2.4	1.8	3.4
Ser	2.4	4.3	2.8	4.7
Glu	14.1	16.1	17	15.0
Gly	5.3	6.8	7.2	7.0
Ala	6.2	8.8	9.5	9.4
Cys	2.9	0	0	1.0
Val	6.8	8.9	8.3	4.8
Met	1.5	1.3	1.8	2.1
Ile	3.6	3.9	4.3	2.4
Leu	8.1	9.7	10.7	12.1
Tyr	3.8	3.8	4.1	3.9
Phe	5.6	5.3	5.6	4.8
His	7.1	5	3.3	3.7
Trp	15.6	nd	nd	nd
Lys	6.1	6.5	7.3	6.4
Arg	nd	3.6	5.1	6.0
Pro	nd	3	0	1.3

The amino acid composition of pea and maize Ft was taken from Laulhere et al. (1988), and the amino acid composition of horse spleen Ft was taken from Rousseau et al. (2008). nd, not determined.

bands with a higher molecular weight could be due to the presence of aggregates either naturally present or generated upon preparation of the sample. Under the experimental conditions of isolation and seed imbibition (24 h) described here, the Fe content in Ft molecule, 200 ± 39 Fe atoms per Ft molecule, was significantly lower than the value previously reported for dry seeds (2 500 Fe atoms per Ft molecule) (Laulhere et al. 1988). These results confirm the quality of the isolated Ft used in the experiments showed below.

Effect of iron chelators on iron loading of soybean Ft

Fe uptake by soybean Ft was evaluated using methods previously described, by measuring the absorbance of oxidized Fe forms at $\lambda = 310$ nm. Native Fe-loaded Ft in the presence of O_2, which is naturally present within chloroplasts, showed a typical Fe loading profile. Initial rates of Fe uptake were determined by linear regression by selecting a linear region of the progress curve during the early period of the reaction, according to Theil et al. (2000) (**Figure 2A**). Since O_2 is required for active Fe uptake, the effect of decreasing O_2 concentration during Fe loading was tested. The Fe solutions and the vessels were sealed with adequate septums, and bubbled with N_2 gas to change O_2 availability. Under these conditions, Fe uptake was decreased by 23, 38, and 62% as compared to control values (without N_2 exposure) after 2, 5 and 10 min of N_2 exposure, respectively. On the other hand, it was previously reported (Bou-Abdallah et al. 2003) that Zn^{2+} binds in the threefold channels of Ft, decreasing the access of Fe^{2+} ions to the ferroxidase centers. Accordingly, Fe incorporation rate was decreased by 24 and 46% by the addition 200 and 400 μM $ZnSO_4$, respectively, and no effect of $ZnSO_4$ was observed over the Fe autoxidation rate (data not shown). These data showed that the isolated Ft function was preserved during the isolation procedure.

Since Fe is available in the cell as part of the LIP, the rate of *in vitro* Fe incorporation into Ft was tested by supplementing the reaction medium with physiological Fe chelators such as citrate, his, and adenosine triphosphate (ATP). The rate observed in the presence of 100 μM Fe chelated to Tris-HCl buffer was not significantly different for the recorded value in the presence of 100 μM Fe-his. However, Fe uptake rate was significantly increased in the presence of Fe-citrate (4.5-fold) or Fe-ATP (14-fold) as compared with the rates observed in the presence of Fe-buffer (**Figure 2B**). Fe autoxidation rate was less than 15% under all conditions.

Generation of $^{\bullet}OH$ during Fe uptake by Ft

Since significant production of H_2O_2 was described during Fe uptake (Zhao 2010), the generation of $^{\bullet}OH$ during the Fe uptake by Ft was tested using electron paramagnetic resonance (EPR) in the presence of DMPO (5,5-Dimethyl-1-pyrroline N-oxide) as spin trap. Both, a Fe^{2+}/Ft molar ratio $< 48{:}1$ (**Figure 3A**), and Fe^{2+}/Ft molar ratio of 1000:1 (**Figure 3B**) were tested to assess $^{\bullet}OH$ production upon the Fe uptake by Ft. In all tested conditions, the EPR signals corresponding to the adduct of $^{\bullet}OH$ with DMPO were observed.

Modification of Ft protein during Fe uptake

The generation of reactive substances in the close environment of the protein could be responsible for protein damage. Thus, both the content of Trp and total thiols were measured upon Fe uptake by Ft. The intrinsic fluorescence of Trp residues of soybean Ft was significantly reduced during Fe uptake in the presence of Fe-citrate, as compared with the decrease observed in the presence of either Fe-buffer of Fe-his (**Figure 4**). Total thiol content in soybean Ft was 23 ± 1 nmol/mg protein, and it was not significantly altered during Fe uptake from Fe-buffer, or Fe-citrate (29 ± 2 and 21 ± 3 nmol/mg protein, respectively).

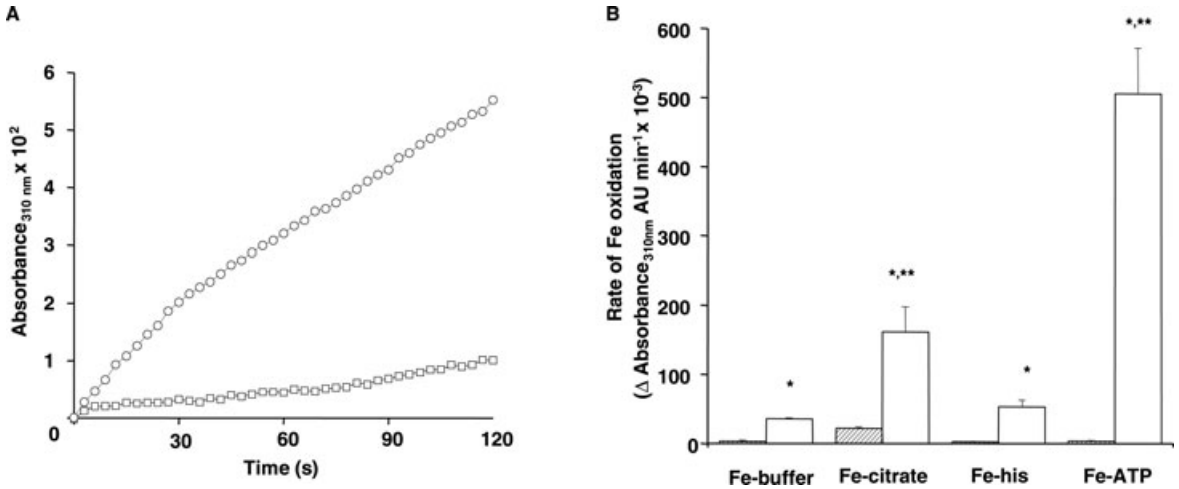

Figure 2. (A) Fe loading into soybean Ft. Kinetic profile of Fe uptake by isolated soybean Ft (○) and Fe^{2+} autoxidation (□). Fe uptake was studied using a Fe^{2+}/Ft molar ratio of 1 000:1 in 10 mM Tris-HCl buffer (pH 5.0) at 25 °C. (B) Effect of Fe chelators on Fe uptake by soybean Ft. SO_4Fe was prepared in 10 mM HCl, and chelated either to citrate (Fe-citrate, 1:2), adenosine triphosphate (ATP) (Fe-ATP, 1:20), or his (Fe-his, 1:5). Fe uptake by soybean Ft (□), and Fe autoxidation (▨).

*significantly different from autoxidation values. Analysis of variance (ANOVA), ($P < 0.05$).

**significantly different from Fe-buffer values. ANOVA, ($P < 0.05$).

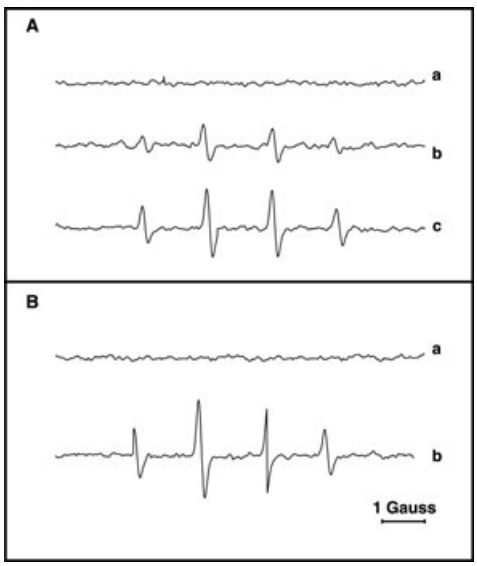

Figure 3. The $^{\bullet}OH$ generation during Fe uptake by isolated soybean Ft.

Fe uptake experiments were performed under the conditions described in Materials and Methods section, with the addition of 0.1 M DMPO (5,5-Dimethyl-1-pyrroline N-oxide) as spin trap.

(A) The generation of $^{\bullet}OH$ during Fe loading into Ft using 0.3 μM Ft, and Fe^{2+}/Ft molar ratio < 48:1: (a) Ft, and DMPO; (b) Ft, 5 μM SO_4Fe, and DMPO; (c) Ft, 10 μM SO_4Fe, and DMPO.

(B) The generation of $^{\bullet}OH$ using 0.1 μM Ft and, Fe^{2+}/Ft molar ratio 1000:1: (a) Ft, and DMPO; (b) Ft, 100 μM SO_4Fe, and DMPO.

Ft ability of preventing $A^{\bullet}$ generation

The supplementation of Ft to homogenates of soybean embryonic axes was analyzed to assess Ft capacity of managing Fe, as a way of preventing $A^{\bullet}$ generation. Homogenates from soybean embryonic axes showed a strong EPR doublet with the spectral features of $A^{\bullet}$ ($a_H = 1.88$ G, $g = 2.0054$) (**Figure 5A**, trace b). The addition to the reaction system with isolated soybean Ft did not affect significantly the $A^{\bullet}$ generation rate (**Figure 5A**, trace c). Fe^{2+} addition (as $FeSO_4$) increased the recorded $A^{\bullet}$ EPR signal by 32% in soybean homogenates (**Figure 5**, trace d). However, the supplementation with isolated Ft (0.3 μM) reduced the recorded signal in the presence of Fe^{2+} to control values (**Figure 5A**, trace e).

Discussion

General features of isolated Ft

Ravet et al. (2009) reported new experimental evidence about Ft role in the defense machinery against oxidative stress, such as acting as a transient buffer for Fe instead of being just an inert storage molecule. This hypothesis opened up a new frame to study Ft participation in multiple oxidative stress conditions in which Fe is a player. To further explore this scenario, Ft was isolated and purified from soybean seeds imbibed for 24 h, before analyzing the nature of the reactions where it is involved. As indicated previously (**Table 1**), the isolated Ft

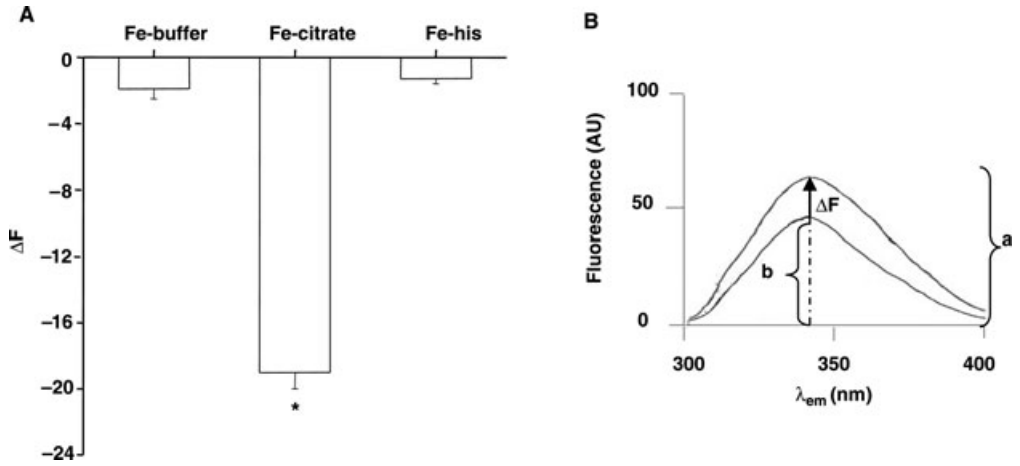

Figure 4. (A) Effect of Fe uptake on the intrinsic fluorescence of Trp. The Trp fluorescence in the Ft molecule was monitored during Fe uptake experiments as described previously, using λ_{exc} = 280 nm, and λ_{em} = 342 nm. (B) Emission spectrum of Trp from isolated soybean Ft was obtained using λ_{exc} = 280 nm. a, fluorescence without any Fe addition, and b, fluorescence with Fe-citrate. ΔF = b–a, after 1 min of incubation.

*significantly different from Fe-buffer values. Analysis of variance (ANOVA), ($P < 0.05$).

showed an amino acid composition compatible with other Ft sources. Moreover, the amino acid composition estimated from the nucleotide sequence of soybean Ft cDNAs reported by Ragland et al. (1990) and by Lescure et al. (1991) was in good agreement with the data shown in **Table 1**. The Trp content seems slightly higher as compared with the predicted amino acid composition probably due to the features of the soybean variety used. The incubation time for isolation was selected considering that Fe is required for the initial development of the seed and thus, the Fe released *in vivo* from Ft would allow the isolation of Ft with a relatively lower Fe content as compared with dry seeds as it was shown here. This fact allowed assessment of the active Fe uptake rates by Ft, as shown in this work. From the data reported here, it was possible to identify the subunits of Ft. However, lower molecular weight subunits could be produced as a consequence of proteolyses processes occurring during the isolation procedure (Sczekan and Joshi 1987) or as products of the *in vivo* Ft degradation by proteinases acting on Ft molecules damaged by free radicals during Fe exchange (Lobréaux and Briat 1991). This observation is consistent with the data presented by Barceló et al. (1997), showing that other smaller subunits of 22–26 kDa appear in other plant Fts, such as pea and alfalfa.

Generation of active species upon Fe uptake by Ft

Grady et al. (1989) and Van Eden and Aust (2001), showed in a chemical medium that the formation of the core in animal Ft may result in a significant production of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) and ultimately $^{\bullet}OH$. Moreover, the $^{\bullet}OH$ formation during Fe^{2+} oxidation by apoferritin (apoFt) was contingent on the ferroxidase activity exhibited by apoFt, and H_2O_2 was shown to be an intermediate product of O_2 reduction (Zhao et al. 2003). Zhao et al. (2006) observed by spin-trapping data that human H-subunit Ft attenuates $^{\bullet}OH$ formation from the Fenton reaction. Recently, Rousseau and Puntarulo (2009) presented data using a biological medium (rat liver homogenate), that showed that radical generation is associated with Ft-dependent Fe release, and moreover that the protein itself was affected during this process. However, the radical-dependent attack to phytoferritin is less clear. Li et al. (2009) have shown a unique pathway for Fe deposition in Ft isolated from plants. According to Zhao (2010) at least three pathways are responsible for Fe deposition in Ft. At a low flux of Fe^{2+} into Ft (<2 Fe^{2+}/H chain), Fe^{2+} oxidation by O_2 is processed completely by the ferroxidase site with an Fe^{2+}/O_2 stoichiometry of 2:1, resulting in the quantitative production of H_2O_2. This reaction is the most important at intermediate Fe loading of the protein (100–500 Fe/protein) (Zhao et al. 2003; Bou-Abdallah et al. 2005). Beyond the 48 Fe^{2+}/protein (2 Fe^{2+}/ferroxidase site) added, some of the H_2O_2 produced at the ferroxidase site rapidly reacts further with Fe^{2+} via a detoxification reaction. This detoxification reaction is in accordance with the protective effect of Ft against oxidative stress. Thus, $^{\bullet}OH$ production was shown using Fe^{2+}/Ft molar ratio <48:1 since H_2O_2 generation is quantitatively relevant. It is also important to point out, that even though $^{\bullet}OH$ production was increased at higher Fe^{2+}/Ft molar ratios, the increase was not linear as compared with Fe^{2+}/Ft molar ratio <48:1. The

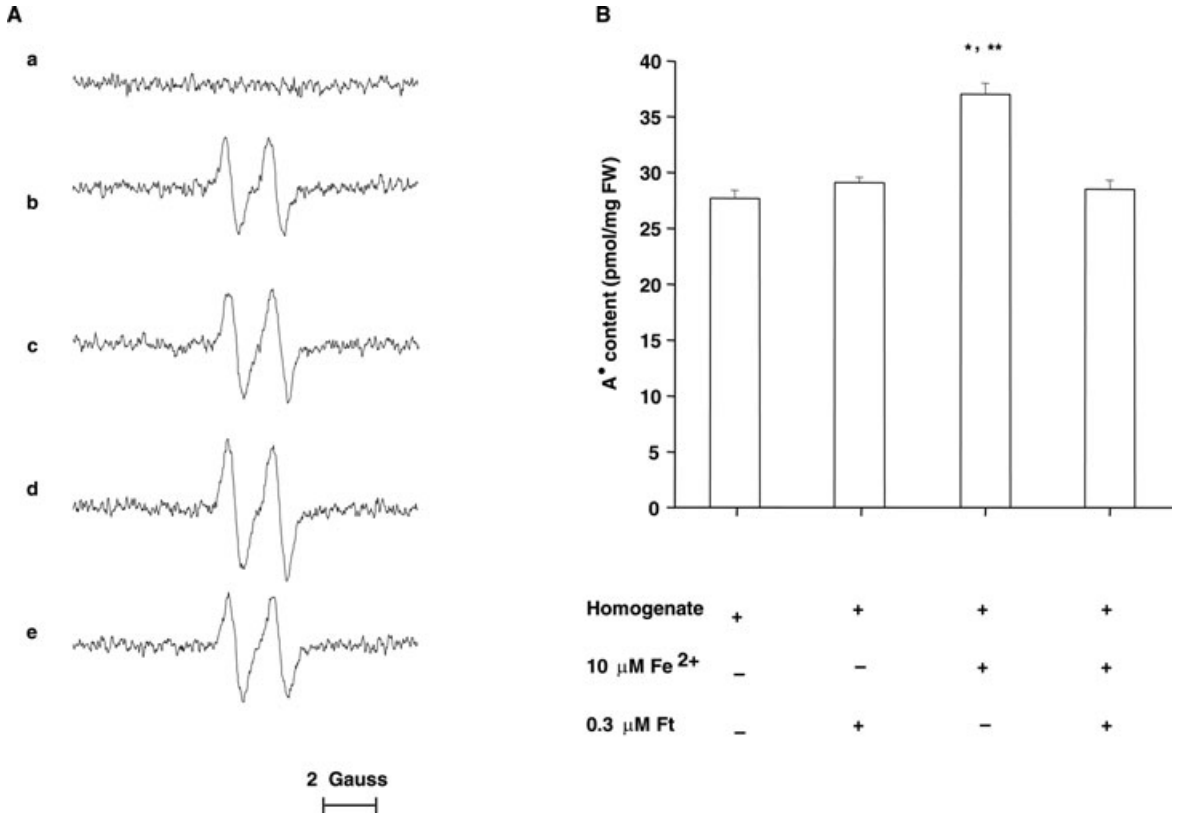

Figure 5. (A) Effect of Ft addition on $A^{\bullet}$ content in homogenates from soybean embryonic axes. After 3 min of incubation at room temperature (25 °C), an equal volume of dimethylsulfoxide (DMSO) was added to stabilize $A^{\bullet}$. (a) 10 mM Tris-HCl buffer, pH = 7.0 + DMSO; (b) homogenates from soybean embryonic axes (125 mg fresh weight (FW)/mL in 10 mM Tris-HCl buffer, pH = 7.0) + DMSO, basal system; (c) basal system in the presence of 0.3 μM Ft; (d) basal system in the presence of 10 μM $FeSO_4$; and (e) basal system in the presence of 10 μM $FeSO_4$, and 0.3 μM Ft. (B) $A^{\bullet}$ content calculated by the integration of the electron paramagnetic resonance (EPR) signals.

*significantly different from the values obtained in the homogenates without any addition. Repeated measures analysis of variance (ANOVA) for paired values, ($P < 0.05$).

**significantly different from the values obtained with homogenates in the presence of $FeSO_4$ and Ft. Repeated measures ANOVA for paired values, ($P < 0.05$).

hypothesis tested here was that Fe uptake from soybean Ft is associated to radical generation, as shown for Ft of animal origin. Bou-Abdallah (2010) reported that Fe chelators can enter and leave the Ft shell during the Fe deposition and release, but the physiological Fe chelates used for loading Fe into Ft are unknown. The reactivity of Fe varies depending on its ligands, therefore, Fe chelation can greatly affect the efficiency of Fe incorporation (Welch et al. 2001), as it was observed under the experimental conditions used in this work. There is very little free Fe^{2+} within the cells ($\sim 10^{-8}$ M), but there is an abundance of potential chelators, such as citrate (Zhao 2010) and ATP (Heldt and Piechulla 2011). In plants, low-molecular-weight chelators, such as nicotianamine, glutathione, phytochelatins, his or citrate, are important metal chelators and often required for long-distance and inter-cellular metal transport, or for the sub-cellular compartmentalization of metals (Krämer et al. 2007).

The Fe concentration used in this study (10 μM), reflects the physiological increase in the LIP in soybean embryonic axes after 24 h of imbibition *in vivo* when supplemented with 500 μM Fe: ethylenediaminetetraacetic acid (EDTA) (Robello et al. 2007). A recent report from Fu et al. (2010) suggested that the release of Fe from phytoferritin could be associated with its degradation mainly originated from the serine protease-like activity of the EP. However, the $^{\bullet}OH$ formation detected under the experimental conditions tested here, suggested that even

upon physiological Fe uptake, damaging effects to the protein itself or to other biomolecules may occur. Masuda et al. (2001) reported that some Trp residues could be positioned in the E helix forming the narrow channels around the fourfold inter-subunit interaction axes. The results presented here showing a decrease in Trp-dependent fluorescence of Ft, are consistent with an alteration in the protein suggesting a deterioration of the Ft structure that could lead to protein degradation. Moreover, from previous data by Van Eden and Aust (2001) (with animal apoFt), and by O′ Connell and Peters (1987) (using liposomes) it was proposed that the Ft protein was modified by both free radical cleavage and addition reactions with aldehyde products of lipid peroxidation.

Hemosiderin is thought to be formed from the aggregation and the partial degradation of the protein shell by lysosomal enzymes (Grady et al. 1989). From the relationship between Ft and hemosiderin it was concluded that hemosiderin peptides are derived from Ft peptides, and that free radical-catalyzed reactions are likely involved in the transformation (O'Connell and Peters 1987). Furthermore, studies of the amino acid sequence of both Ft and hemosiderin showed that hemosiderin has a relatively low abundance of thiol and aromatic residues, residues that are likely to react with $^{\bullet}OH$ (O'Connell and Peters 1987). The data presented here show that, under the conditions tested here, Trp residues were affected during Fe uptake, altering the protein primary structure. Clearly, radicals produced during Fe uptake from Ft in the presence of Fe^{2+} within the environment of the shell of the Ft, may result in the oxidation and fragmentation of the nearby amino acids. However, it is unclear to what extent radical production in the cell contributes to hemosiderin formation since it depends on Fe^{2+} and O_2 concentration and their flux in the environment of the Ft.

Protective effect of Ft

However, it is critical to obtain an integrative picture of the cellular situation by taking into account Ft's central role as a cytoprotective protein, since its main function is to serve as a Fe storage depot, as supported by several *in vivo* experiments with cells and animals (Galatro et al. 2007). In this regard, the data reported here showing that $A^{\bullet}$ generation by soybean homogenates was significantly decreased by the addition of Ft, under the appropriate experimental ratios of Fe:Ft, may be relevant to understanding Ft function at the cellular level. It might not be just as an inert storage Fe protein, but also it could be involved in the generation of free radical species by Fe-catalyzed reactions (such as $^{\bullet}OH$). Moreover, the scavenging of the cellular produced radicals, by reacting with Ft itself leading to damage, or by the prevention of radical generation through the Fe uptake, are Ft functions to be considered. These features may be critical when analyzing secondary effects linked to several *in vivo* conditions involving Fe metabolism. Since Ft remains a central protein of Fe homeostasis, crucial aspects of its biological functions need to be clarified to obtain new insights on many disorders in plant development.

Materials and Methods

Ft isolation

Soybean seeds (*Glycine max*, var DM 4800) were soaked for 24 h in tap water in the dark under controlled temperature conditions (26–28 °C), and Ft was isolated according to Robello et al. (2007) with modifications. Seeds were homogenized in two volumes of extraction buffer (50 mM potassium phosphate, 2% (w/v) polyvinylpyrrolidone, 20 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, pH 7.0). The homogenate was centrifuged for 15 min at 16 16 266g at 4 °C and 0.7% (w/v) $MgCl_2$ was added to the supernatant. This supernatant was kept at 0 °C for 30 min and then was centrifuged at 2 988*g* for 15 min at 4 °C. This second supernatant was filtered through 53 μm nylon filter and was added with trisodium citrate at a final concentration of 2% (w/v). Finally, this suspension was ultracentrifuged at 250 000g through 5 mM potassium phosphate buffer, 0.01% (v/v) Triton X-100, 50% (v/v) glycerol, pH 7.0 at 4 °C for 1 h. The obtained brown pellets were resuspended in 10 mM Tris-HCl buffer, 0.15 M NaCl, pH 7.0. The non-solubilized material was eliminated by centrifugation at 10 000g for 15 min. The supernatant containing Ft was then loaded on a Sephacryl S-300 (100 × 1.6 cm) column previously equilibrated in 10 mM Tris-HCl buffer, 0.15 M NaCl, pH 7.0. Fractions were collected and protein and Fe (protein bound Fe) were estimated spectrophotometrically at $\lambda = 280$ and 420 nm (Bejjani et al. 2007), respectively. The Fe-rich fractions were pooled and protein content in the sample was measured according to Bradford (1976) using bovine albumin (Sigma) as standard.

Amino acid composition of isolated Ft

After purification, the protein sample was concentrated by centrifugation and vacuum at room temperature. Then the sample was hydrolyzed in 0.2% tryptamine in metasulphonic acid (0.5 mL/mg nitrogen in the sample) for 18 h at 110 °C according to Fountoulakis and Lahm (1998). After setting the pH sample to 2.2 using NaOH 10% (v/v), the sample was filtered through Teflon membranes (0.2 μm, Millipore) and put into glass vials in the presence of L-norleucine (0.25 mM) as internal standard, in citrate buffer 0.2 M pH 2.2. The samples were separated by ion-exchange chromatography, using a PEEK column (Ultropac 8) with a pH gradient between 2.2 a 4.3, and the initial and final temperatures were 37 and 135 °C respectively, according to Spackman et al. (1958). Once the

amino acids had been separated, their respective quantities were determined by adding ninhydrin, measuring its derivatives spectrophotometricaly at $\lambda = 570$ nm, according to Le Boucher et al. (1997).

SDS-PAGE and western blot analysis of Ft

Samples were prepared in 40 mM buffer Tris-HCl pH 6.8 containing 10% (v/v) glycerol, 1% (w/v) SDS, 1% (v/v) β-mercaptoethanol and 0.005% (w/v) bromophenol blue, and were separated by electrophoresis in 15% (w/w) SDS-polyacrylamide gel under denaturating conditions. Gels were either stained with coomassie blue to assess the purity of the isolated Ft, or layered onto a nitrocellulose membrane 0.2 μm (BioRad, Hercules, CA, USA) to transfer the proteins by electroblotting. The nitrocellulose membranes were incubated with anti-soybean Ft primary antibody developed in mouse (1/2 000 dilution) and followed by a horseradish peroxidase-conjugated goat anti-mousse secondary antibody (Jakson, West Grove, PA, USA) (1/10 000 dilution). Finally the blots were developed with an Inmuno-StartTM HRP chemiluminescent kit (BioRad).

Production of antibodies against soybean seed Ft

Antibodies against the isolated soybean Ft were obtained in mouse, that were injected with the bands experimentally obtained corresponding to 26–28 kDa molecular weight cut from the gel stained with coomasie blue. The titer of the soybean seed Ft antibody in the mouse serum was determined after the second injection, prior to final bleeding.

Total Fe content in Ft

Samples were mineralized using HNO_3 40% (v/v) and Fe content was determined after reduction with thioglycolic acid measuring the absorbance at $\lambda = 535$ nm in the presence of bathophenanthroline (Brumby and Massey 1967).

Fe uptake from Ft

Fe uptake was studied using a Fe^{2+}/Ft molar ratio of 1 000:1 (0.1 mM $FeSO_4$ and 0.1 μM Ft) according to Masuda et al. (2001). The reaction was performed in 10 mM Tris-HCl buffer (pH 5.0) at 25 °C. Fe incorporation was monitored measuring the absorbance of oxidized Fe forms at $\lambda = 310$ nm (Treffy et al. 1995; Masuda et al. 2001) using $FeSO_4$ prepared in 10 mM HCl, and chelated either to citrate (Fe-citrate, 1:2), ATP (Fe-ATP, 1:20), or his (Fe-his, 1:5) prepared in 10 mM Tris-HCl buffer (pH 5.0), as it is indicated in each experiment. Fe chelates were prepared immediately before addition to the reaction media containing Ft.

Hydroxyl radical ($^{\bullet}$OH) generation during Ft Fe uptake

Generation of $^{\bullet}$OH was detected by EPR using a Bruker (Karlsruhe, Germany) ECS 106 spectrometer operating at 9.74 GHz with 50 kHz modulation frequency, 20 mW power, 0.9 G modulation amplitude, 3 480 G center field, conversion time 40.96 ms, 327.28 ms time constant (Rousseau and Puntarulo 2009). All spectra were recorded approximately 1 min after the addition of 0.1 mM Fe^{2+} to 0.1 μM Ft in 10 mM Tris-HCl buffer (pH 5.0) at 25 °C, except where otherwise indicated. The $^{\bullet}$OH was trapped by DMPO at a final concentration of 0.1 M.

Tryptophan fluorescence

Intrinsic fluorescence the Ft molecule dependent on Tryptophan (Trp) presence was monitored during Fe uptake by recording fluorescence at $\lambda_{exc} = 280$ nm, and $\lambda_{em} = 342$ nm, according to Welch et al. (2001).

Thiol content

Using the reaction medium described previously for Fe uptake experiments, the Ft was analyzed to evaluate total thiol content according to Sedlak and Lindsay (1968), using *5,5′*-dithiobis-(2-nitrobenzoic acid) (DTNB), and measuring the absorbance a $\lambda = 412$ nm. Glutathione was used as standard.

Ascorbyl radical ($A^{\bullet}$) generation in homogenates from soybean axes

Homogenates from soybean embryonic axes (125 mg FW/mL in 10 mM Tris-HCl buffer, pH 7.0) were incubated for 3 min at 25 °C (basal reaction system) alone, in the presence of 0.3 μM Ft, or in the presence of 10 μM $FeSO_4$ with or without 0.3 μM Ft, and then the same volume of dimethyl sulfoxide (DMSO) was added. $A^{\bullet}$ was detected by EPR using a Bruker ECS 106 spectrometer operating at 9.73 GHz with 50 kHz modulation frequency, 10 mW microwave power, 1 G modulation amplitude, 3 487 G center field, 81.92 ms conversion time, and 327.68 ms time constant (Robello et al. 2007). Signal quantification was performed using an aqueous solution of 2,2,5,5-tetramethyl piperidine-1-oxyl (TEMPO) 1.96 mM. EPR spectra for both sample and TEMPO solutions were recorded at exactly the same spectrometer settings and the first derivative EPR spectra were double integrated to obtain the area intensity, then the concentration of $A^{\bullet}$ was calculated according to the TEMPO concentration used.

Statistical analyses

Experiments were carried out in duplicate and replicated with at least three different preparations. Unless otherwise indicated, data in the text and figures are expressed as mean ± standard

error of the mean (*SEM*). Statistical tests were carried out using GraphPad Instat® for Windows, GradPad software, Inc. version 3.01, analysis of variance (ANOVA), Bonferroni post test.

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