Biometals (2011) 24:225–237
DOI 10.1007/s10534-010-9388-z

A role for ferritin in the antioxidant system in coffee cell cultures

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Received: 15 February 2010 / Accepted: 22 October 2010 / Published online: 3 November 2010

Abstract Iron (Fe) is an essential nutrient for plants, but it can generate oxidative stress at high concentrations. In this study, *Coffea arabica* L. cell suspension cultures were exposed to excess Fe (60 and 240 μM) to investigate changes in the gene expression of ferritin and antioxidant enzymes. Iron content accumulated during cell growth, and Western blot analysis showed an increase of ferritin in cells treated with Fe. The expression of two ferritin genes retrieved from the Brazilian coffee EST database was studied. *CaFER1*, but not *CaFER2*, transcripts were induced by Fe exposure. Phylogenetic analysis revealed that *Ca*FER1 is not similar to *Ca*FER2 or to any ferritin that has been characterised in detail. The increase in ferritin gene expression was accompanied by an increase in the activity of antioxidant enzymes. Superoxide dismutase, guaiacol peroxidase, catalase, and glutathione reductase activities increased in cells grown in the presence of excess Fe, especially at 60 μM, while the activity of glutathione *S*-transferase decreased. These data suggest that Fe induces oxidative stress in coffee cell suspension cultures and that ferritin participates in the antioxidant system to protect cells against oxidative damage. Thus, cellular Fe concentrations must be finely regulated to avoid cellular damage most likely caused by increased oxidative stress induced by Fe. However, transcriptional analyses indicate that ferritin genes are differentially controlled, as only *CaFER1* expression was responsive to Fe treatment.

Keywords *Coffea arabica* · Ferritin · Oxidative stress · Iron toxicity · Antioxidant enzymes

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Introduction

Iron (Fe) is an essential mineral for animals and plants. Iron deficiency in humans (anaemia) affects about 30% of the world population (i.e. about two billion people), with the highest prevalence found in

developing countries, where vegetable-based diets are the primary food source (Goto et al. 1999, 2001; Grotz and Guerinot 2006; Haas et al. 2005).

In plants, Fe is required for essential life-sustaining processes such as respiration and photosynthesis, where it participates in electron transfer through reversible redox reactions and cycling between Fe^{2+} and Fe^{3+} (Kim and Guerinot 2007). Fe is also a constituent of a number of important macromolecules involved in DNA synthesis and metabolism (Ravet et al. 2009). Fe-deficient plants typically display interveinal chlorosis in young leaves and a dramatic decrease in productivity (Donnini et al. 2009). This characteristic symptom is the consequence of decreased chlorophyll content in proportion to carotenoids (Briat et al. 1995).

Whereas Fe deficiency results in chlorosis, Fe excess is believed to generate oxidative stress (Kampfenkel et al. 1995) in the presence of O_2 or H_2O_2 (Briat and Lebrun 1999). Transition metals such as Fe frequently have unpaired electrons, which may react with oxygen to generate harmful free radicals by the Fenton reaction (Briat and Lebrun 1999; Ravet et al. 2009). Hydroxyl radicals ($^{\cdot}OH$) generated by this reaction are powerful oxidising agents of biological molecules, leading to major cellular damage and cell death (Briat and Lebrun 1999; Strozycki et al. 2003). However, the controlled generation of reactive oxygen species (ROS) may be a part of plant defence systems against pathogens (Strozycki et al. 2003). Thus, Fe homeostasis must be strictly controlled (Briat and Lebrun 1999).

The protein ferritin is known to modulate Fe concentrations to the levels required by cells in a soluble and biologically available form, avoiding Fe overload toxicity (Wei and Theil 2000). Ferritin is a ubiquitous Fe-binding protein that plays an essential role in cellular Fe homoeostasis as part of a multimeric complex that can store up to 4,500 Fe atoms in the central cavity (Briat et al. 2009; Petit et al. 2001). Ferritins are distributed across a family composed of three sub-families, the classical ferritins (Ftn), the bacterioferritins (Bfr) and the "DNA-binding proteins from starved cells" (Dps proteins), also known as miniferritins. The Ftn and Bfr proteins share high similarity and function in Fe storage. The Dps ferritins are more distinct and play two roles in oxidative protection: binding ferrous ions and non-specifically binding DNA to shield it from oxidative radicals (Andrews 2010; Haikarainen and Papageorgiou 2010). The Ftn proteins are the archetypical members of the ferritin family, present in all three domains of life, whereas the Bfr proteins are limited to the bacterial and archaeal domains (Andrews 2010). The Dps proteins are well-conserved prokaryotic proteins and have a lower Fe-storage capacity than the other ferritins (Zhao et al. 2003; Andrews 2010). In addition, the Dps proteins have other distinctive features compared to the other members of the ferritin family. Whereas Ftn ferritins use O_2 as the Fe oxidant in the production of H_2O_2, the Bfr proteins can use both O_2 and H_2O_2, and Dps ferritins preferentially use H_2O_2, which is more efficient in Fe oxidation than O_2 (Chiancone and Ceci 2010).

In plants, the ferritin gene encodes an *N*-terminal target peptide that delivers the protein to the plastid, the organelle in which it preferentially accumulates (Wei and Theil 2000). However, ferritin has also been identified in *P. sativum* and *A. thaliana* mitochondria (Zancani et al. 2004). Some studies have shown that ferritins accumulate predominantly in non-green plastids, such as etioplasts or amyloplasts, while low levels of protein were found in chloroplasts where photosynthetic is active (Briat et al. 1999). Plant ferritin is usually found in small amounts in plastids of vegetative organs, such as roots and leaves, but it accumulates in seeds during embryo maturation (Briat et al. 1995; Déak et al. 1999). Moreover, excess Fe induces an increase in the transcription of the ferritin gene in soybean, with a corresponding increase in the accumulation of the protein (Lescure et al. 1991).

The levels of the ferritin proteins respond to changes in Fe concentration and O_2 or oxidant agents in plants, animals, and bacteria (Hintze and Theil 2006). In addition to their role in Fe storage, some ferritins are also involved in the detoxification of Fe, O_2, and H_2O_2 under certain conditions (Boughammoura et al. 2007). Animal ferritins are composed of two subunit types, H and L (Orino et al. 2001). Ferritin is found in the cytoplasm and mitochondria of animal cells, and unlike plant ferritin genes, which are regulated only at the level of transcription, cytoplasmic animal cell ferritin synthesis is controlled during both transcription and the translation (Tsuji et al. 2000; Hintze and Theil 2006). In animals the regulation of mRNA transcription by Fe depends on the non-coding iron-responsive element (IRE) and

IRE-binding proteins (IRPs), which are ferritin mRNA repressors and inhibit translation when cellular iron levels are low (Hintze and Theil 2005). Ferritin gene expression responds to antioxidants and oxidant inducers, and an antioxidant-responsive element (ARE) sequence was reported in the mouse ferritin-L and -H genes (Hintze and Theil 2005, 2006). This ARE is found in the promoter of diverse antioxidant response and detoxification genes and induces the expression of many proteins involved in defence against oxidative stress, such as thioredoxin reductase, thioredoxin, quinone reductase, heme oxygenase and glutathione (Hintze and Theil 2005, 2006). In bacteria, ferritin is located is the cytoplasm and DNA complex, and its synthesis is controlled at the transcriptional level (Hitze and Theil 2006).

The ability of ferritins to store excess free Fe enables them to protect cells against the toxic effects of Fe and make ferritins an important component of the antioxidative stress response in plants (Briat and Lebrun 1999; Ravet et al. 2009; Wei and Theil 2000). Plants have developed both non-enzymatic and enzymatic antioxidant defences against ROS (Gratão et al. 2005, 2008), but the major factor responsible for Fe homeostasis control is ferritin (Strozycki et al. 2003).

Coffee is an economically important crop grown in Brazil, the world leader in coffee bean production. The economic importance of coffee can be also measured by the fact that from the field to final product commercialisation, the whole "coffee chain" employs 25 million people (Lin et al. 2005). Recently, a Brazilian Coffee Genome Project (BCGP) sequenced more than 200,000 ESTs from 37 cDNA libraries of three different species, resulting in approximately 33,000 distinct unigenes (Vieira et al. 2006). Using information generated from the Coffee EST databank, this work investigated whether excess Fe induces an oxidative stress condition in coffee cells, leading to an antioxidative stress response. Coffee cell suspensions exposed to Fe excess were used as a model system to investigate the expression of ferritin and the enzymatic antioxidant response. To date, a few studies have been performed on the coffee enzyme antioxidant response to metal toxicity (Gomes-Junior et al. 2006a, 2006b, 2007), but as far as we are aware, Fe homeostasis control in this plant has not been previously investigated.

Materials and methods

Cell suspension

Leaves of the third and fourth leaf pairs of *C. arabica* L. var. Mundo Novo (IAC 388-1) were used to produce the cell cultures. The explants were maintained in CIM (callus inducing medium) solid medium (Neuenschwander and Baumann 1992), pH 5.8, containing MS salts (Murashige and Skoog 1962) and supplemented with 10 mg/L thiamine–HCl, 100 mg/L inositol, 30 g/L sucrose, 4 mg/L kinetin, and 1 mg/L 2,4-D. Calluses produced by 12–13 weeks in the dark and presenting a pale-yellow colour and a friable aspect were selected, transferred to 30 mL liquid CIM medium in 250 mL Erlenmeyer flasks and maintained at 100 rpm in the dark at 25 ± 2°C. Every week, half the volume of each flask was transferred to a new flask containing 15 mL CIM medium. At this stage, large aggregates were eliminated using forceps to obtain cell suspensions formed by small homogeneous aggregates. All of these procedures were carried out in a sterile laminar-flow hood.

Treatment of the cells

To induce the synthesis of ferritin, the liquid medium of flasks containing 7-day-old coffee cells was suction-dried and approximately 4 g cells were transferred to a new flask containing 50 mL of liquid CIM medium to which was added 0.0 (control), 300, or 1,200 μM ferrous sulphate ($FeSO_4 \bullet 7H_2O$), corresponding to Fe concentrations of 20, 60, and 240 μM, respectively. Cell cultures were grown for 72 h (100 rpm in the dark, 25 ± 2°C) and then harvested. At this stage, the cells were in the exponential growth phase (Filippi et al. 2007). Cells were recovered in a Buchner filter under pressure, washed with 500 mM NaCl and then distilled water, immediately frozen in liquid nitrogen and stored at –80°C for further analyses.

Analysis of the Brazilian coffee EST database

Coffee ESTs encoding ferritin genes were retrieved from the coffee EST database (Vieira et al. 2006) using "ferritin" as the key word. From 91 EST sequences retrieved, 25 were from cDNA libraries

of stress conditions. A complete consensus mRNA sequence was obtained from these 25 ESTs and used for molecular studies. This was designated *CaFER1* (GenBank GQ913984). *CaFER1*. A second complete coding region consensus sequence, labelled *CaFER2* (GenBank GU001880), was also obtained from searches at the Coffee EST database using *Arabidopsis thaliana* ferritin homologs.

Total RNA isolation and cDNA synthesis

Cells were ground to a fine powder in liquid N_2 using a pre-cooled mortar and pestle and then c.a. 400 mg was extracted with 2.7 mL TRIzol reagent (Invitrogen). The extraction procedure followed the protocol described by Puthoff et al. (2005). Total RNA was quantified at 260–280 nm and a sample loaded onto 1.0% agarose gel with ethidium bromide for a quality inspection under UV. Five micrograms of total RNA was treated using DNAse-free (Ambion), and the first-strand cDNA synthesis was carried out using Superscript III (Invitrogen), both procedures following the manufacturer's instructions.

PCR, cloning and sequencing

Primers for *CaFER1* were designed with Primer3 software (http://frodo.wi.mit.edu/) (Forward: 5′-GATCCCCTGCTTCAAGAATTT-3′; Reverse: 5′-AGTCTGAAAAGCCGCACACT-3′). RT–PCR with these primers produced a single fragment of 1110 bp. All PCR reactions were performed under the following conditions: 94°C for 3 min, 35 cycles of 94°C for 45 s, 55°C for 30 s, 72°C for 60 s. The amplified fragments were purified from agarose gels using the Illustra GFX PCR and Gel Band Purification kit (GE Healthcare), inserted into pGEM-T easy vector (Promega), cloned in *Escherichia coli* strain DH10b and then sequenced (3100 Genetic Analyser—Applied Biosystems) using the kit DYEnamic (GE) sequencing kit and T7 and SP6 as primers. The sequence was confirmed in the Coffee EST database using the software ClustalX 1.83. Only *CaFER1* was sequenced due to its relationship with stress library conditions constructed for the Brazilian Coffee EST database.

Quantitative real-time PCR

Polymerase chain reactions were carried out in an optical 96-well plate with an ABI Prism 7500 Sequence Detection System (Applied Biosystems) using SYBR® green PCR Master Mix (Applied Biosystems) to monitor dsDNA synthesis. Primers specific for *CaFER1* (Forward: 5′-CTGAAAAGCCGCACACTGC-3′; Reverse: 5′-TTGTTGGTATTTTCCTCATCTCAA-3′) and *CaFER2* (Forward: 5′-CATGCCCTGTTTGCCTATTT-3′; Reverse: 5′-GCAAGACCCTTGAGAGCAAC-3′) were designed using primer express 2.0 software (Applied Biosystems) with default parameters. PCRs were performed as previously described (Salmona et al. 2008). Cycle conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 94°C for 15 s and 60°C for 1 min. The PCR efficiency was better than 90% for *CaFER1* and *CaFER2* as estimated by LinReg Software (Ramakers et al. 2003). Confirmation of amplicon specificity was based on the dissociation curve at the end of each run. In order to obtain an accurate internal normalisation, the expression stability of five putative housekeeping genes (ribosomal protein rpl39—[Forward: 5′-GAACAGGCCCATCCCTTATTG-3′; Reverse: 5′-CGGCGCTTGGCATTGTA-3′] (Lepelley et al. 2007); actin—[Forward: 5′-GGGAAATTGTCCGTGACATC-3′; Reverse: 5′-GGCTGTTTCAAGCTCTTGCT-3′]; ribosomal protein S19—[Forward: 5′-CCGAATGCCATTTTTGTCTT-3′; Reverse: 5′-TCCAAACCCAGTTGACTTGC-3′]; glyceraldehyde 3-phosphate dehydrogenase—[Forward: 5′-AGGCTGTTGGGAAAGTTCTTC-3′; Reverse: 5′-ACTGTTGGAACTCGGAATGC-3′]; cyclophylin—[Forward: 5′-CGTATCACGGAGGGTATGGA-3′; Reverse: 5′-GAAGACCCAACCTGCTCAAC-3′]) was investigated using the geNorm software (Vandesompele et al. 2002). Of these candidates, ribosomal protein rpl39 was the most stable and was used as an endogenous control. Each gene reaction was performed in triplicate, and PCR reactions in the absence of template were also performed as negative controls for each primer pair. Data were analysed using the threshold cycle (Ct), which is the fractional cycle number at which a fixed amount of DNA is formed. The relative gene expression was presented using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Cellular Fe concentration measurement

The Fe content was determined in coffee cells that were dried at 60°C and ground to a powder in mortar by subjecting the pulverised material to inductively coupled plasma-optical emission spectroscopy (ICP-OES; JobinYvon, JY50P) after HNO_3–$HClO_4$ digestion.

Phylogenetic analysis

Phylogenetic analysis of *CaFER1* and *CaFER2* was performed using MEGA 4 software (Kumar et al. 2008b). The predicted proteins were aligned with the ClustalW program included in MEGA 4 and then used to construct a phylogenetic tree using the neighbour-joining method (Saitou and Nei 1987). Gap regions were excluded by manual adjustments to avoid ambiguities in regions with problematic alignments. The Poisson model was used to provide unbiased estimates of the number of substitutions between sequences. Bootstrap values were obtained after 1,000 replications. The accession numbers of the sequences analysed were *Arabidopsis thaliana*: Fer1—CAA 63932.1, Fer2—CAC85498.1, Fer3—CAC85399.1, Fer4—CAC85400.1; *Glycine max*: Fer1—P19976, Fer2—Fer3 Acc. Q948P6, Fer4—Q948P5; *Oryza sativa*:—AAK53812.1; *Zea mays*: Fer1—P29036, Fer2—P29390; *Nicotiana tabacum*: Fer1—Q8R X97.1, Fer2—Q8H1T3.1; *Lycopersicon esculentum*: BE431630 (translated amino acid sequence); *Pyrus pyrifolia*: ABD66597.1; *Populus trichocarpa*: XP_00 2323610.1; *Solanum tuberosum*: ABU49726.1; Ferritin heavy chain—AAI05803.1 and ferritin light chain—AAA52439.1 of *Homo sapiens* were used as an external group.

Protein extraction and Western blot analysis

Coffee cells were homogenised in 5 mL of 100 mM HEPES, pH 7.0, 2 mM EDTA, 5 mM $MgCl_2$, 2% ascorbic acid, and 10 mM 2-mercaptoethanol. The homogenate was incubated for 30 min on ice, centrifuged (27,000 × *g* for 25 min at 4°C) and then filtered on PD10 Sephadex G25 columns (Amersham Biosciences). Proteins were eluted with 20 mM HEPES, pH 7.0, and total soluble protein was quantified (Bradford 1976) using bovine serum albumin (Sigma) as a standard. For Western blot analysis, 20 μg total soluble proteins from cells was separated on 12% SDS polyacrylamide gels and then transferred by electrophoresis to nitrocellulose membranes (Hybond C, GE Healthcare) using a horizontal semi-dry electroblot apparatus (GE Healthcare). After blocking membranes (PBS—phosphate-buffered saline: 100 mM Na_2HPO_4, 17 mM KH_2PO_4, 1.4 M NaCl, 27 mM KCl—plus 5% skim milk, 0.02% sodium azide and 0.02% Tween 20) at room temperature for 2 h, blots were incubated in primary polyclonal antibody (anti-ferritin) in blocking solution, at a 1:100 dilution overnight. Polyclonal anti-ferritin antibody was kindly supplied by Dr Janette Palma Fett (Universidade Federal do Rio Grande do Sul) (Silveira et al. 2009). The membrane was washed three times with PBS and then incubated in secondary antibody anti-rabbit conjugated with alkaline phosphatase (Sigma–Aldrich) at a 1:300 dilution in 150 mM NaCl, 50 mM TRIS–HCl, pH 7.5, plus 5% skim milk for 1 h. The membranes were washed in 150 mM NaCl, 50 mM TRIS–HCl, pH 7.5, and ferritin bands were detected using 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium substrates (Sigma–Aldrich). Band intensities relative to controls were obtained using the ImageJ software (http://rsbweb.nih.gov/ij/).

Antioxidant enzyme activities

The samples were extracted as previously described for the western blot analysis and stored at –80°C until activity analyses of superoxide dismutase (SOD, EC 1.15.1.1), guaiacol peroxidase (GPOX, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), glutathione *S*-transferase (GST, EC 2.5.1.18) and glutathione reductase (GR, EC 1.6.4.2) were performed. CAT activity was determined as described by Azevedo (1998). GR and GPOX activities were determined as described by Gomes-Junior et al. (2006a). Non-denaturing PAGE was carried out for SOD activity staining (Gomes-Junior et al. 2006b). GST activity was determined as described by Habig and Jakoby (1981).

Statistical analysis

A one-factor analysis of variance (ANOVA) was used to assess differences in *CaFER1* and *CaFER2* gene expression between treated and non-treated cells as well as to analyse CAT, GR, GOPX and GST

activities and western blot results. For Fe content and enzyme activities, three technical replicates were obtained for each of three biological replicates of each sample. For Western blot analyses, only one technical replicate was made for each of three biological replicates. When a significant variation was found, Tukey's test was used as a post hoc comparison to adjust *P* values. For Fe accumulation, data were expressed as standard error of the mean (±SEM). Statistical significance was considered at an α of 5%. Analyses were carried out using the BioEstat 3.0 program (Ayres et al. 2003).

Results

Sequence characterisation of the CaFER1 gene

The sequence obtained for *CaFER1* is 1110 nucleotides long and contains an open reading frame of 289 amino acids, which is preceded by 47 nucleotides before the first methionine, and 193 nucleotides are present downstream of a TAG stop codon at position 870. This open reading frame encodes a peptide that has a predicted molecular mass of 32.1 kDa. Full nucleotide and deduced amino acid sequences are shown in Fig. 1. The cDNA of *CaFER1* was sequenced and showed high similarity to other ferritin gene sequences in the GenBank and 100% identity with the ferritin in the Coffee EST database, which was the basis for the design of the primers.

Phylogenetic analysis

A phylogenetic analysis was carried out with the deduced amino acid sequences of *CaFER*1, *CaFER*2 and other ferritin genes. *Ca*FER1 has similarity with few characterised ferritins, grouping with *P. pyrifolia*, *P. trichocarpa*, *G. max* FerH1 and FerH2, and *At*FER1 (Fig. 2). *Ca*FER2 exhibited high similarity

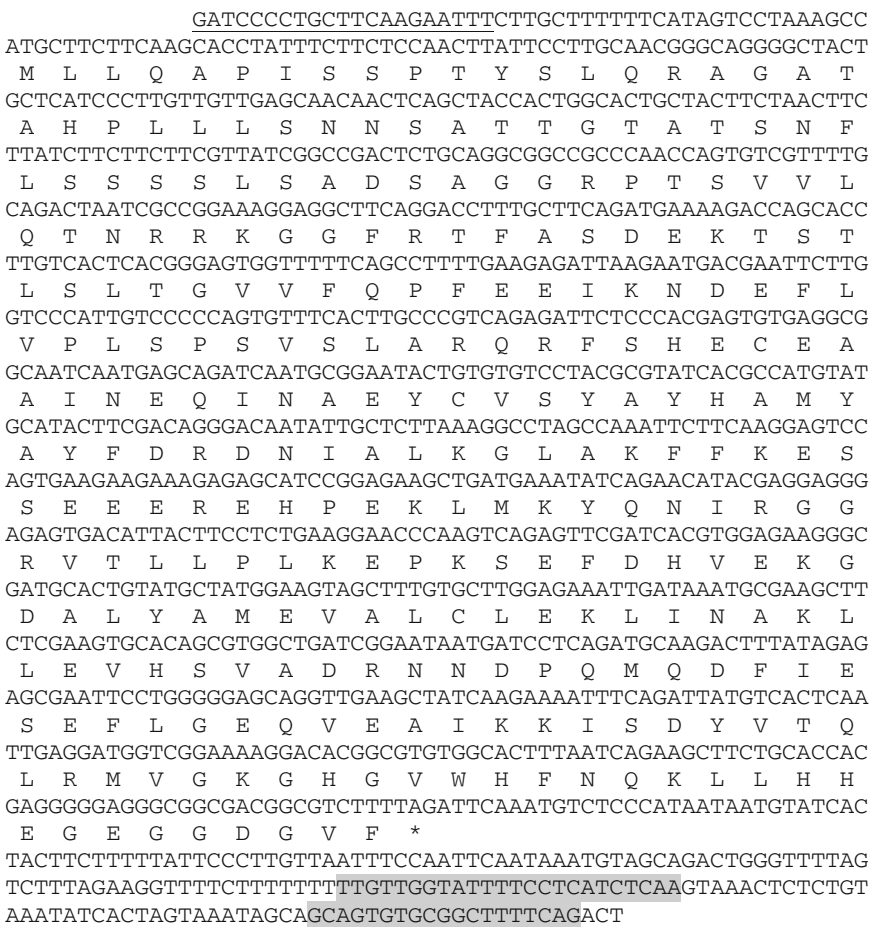

Fig. 1 Nucleotide and deduced amino acid sequences of *CaFER1*. The stop codon is denoted by an *asterisk*. *Underlined* nucleotides indicate the RT–PCR primers and *grey* colour nucleotides are the qPCR primers

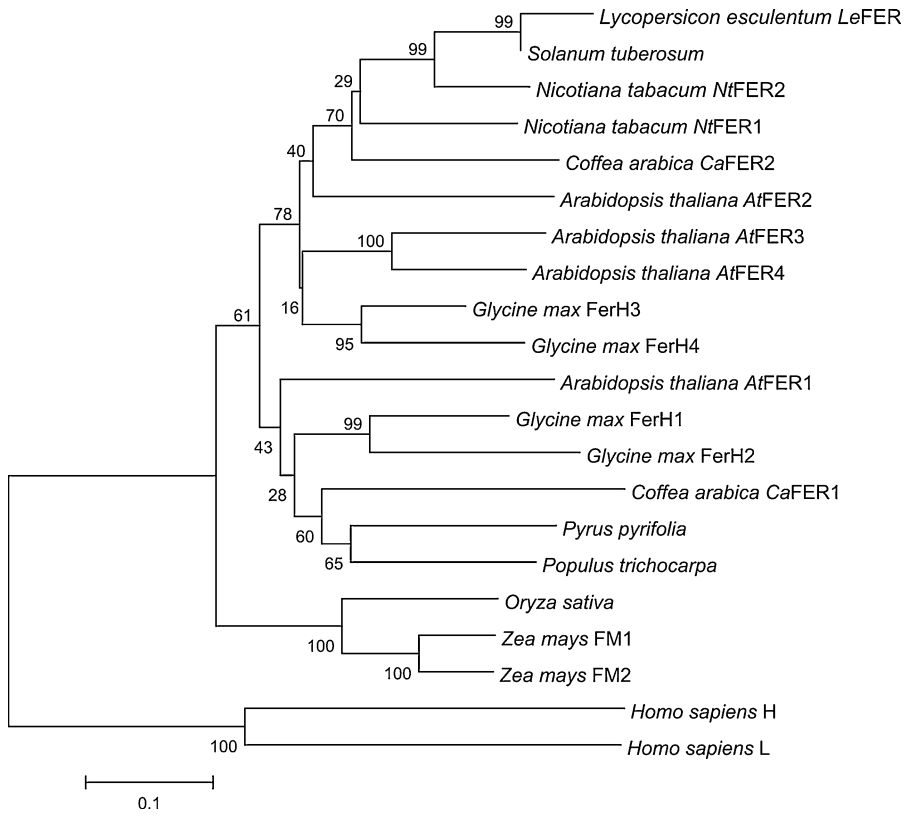

Fig. 2 Phylogenetic tree of plant ferritin amino acid sequences. The percentages of bootstrap support are shown in the branches. These values were calculated from 1,000 bootstrap re-samplings. Nodes supported by bootstrap values higher than 50% are shown. Two human ferritins were added to the tree as external group

with *N. tabacum* *Nt*FER1 and *Nt*FER2, *L. esculentum* *Le*FER, and the ferritin of *S. tuberosum*. In addition, *Ca*FER2 also exhibited a higher degree of similarity with *At*FER2 compared to other ferritins of *A. thaliana* (Fig. 2). The highest BLAST score for *Ca*FER2 was observed with *S. tuberosum* ferritin (2e-104). *Ca*FER1 showed low similarity with *Le*FER (identity = 62%, BLAST score = 3e-49).

RT–qPCR and Western blot analysis

CaFER1 expression was up-regulated by Fe supplementation, as the transcript levels were significantly increased in the coffee cells ($P < 0.05$, Fig. 3a). The expression of *CaFER1* mRNA showed approximately 8.4- and 15.8-fold increases with 60 and 240 μM Fe supplementation, respectively, compared to the control treatment. No significant difference was observed for *CaFER2* expression in the Fe treatments ($P = 0.7546$, Fig. 3a).

Western blot analyses showed a significant increase of the level of ferritin protein in cells treated with Fe (Fig. 3b and c), indicating that *CaFER1* expression is positively correlated to protein levels, suggesting transcriptional control induced by Fe.

Iron content

Fe accumulation in suspension cells of *C. arabica* increased significantly with Fe supplementation, especially at 240 μM, in which the Fe concentration was over fourfold higher than that detected in the 60 μM Fe treatment (Fig. 3d).

Enzyme activities

Activities of antioxidant enzymes were altered by excess Fe (Fig. 4). In general, activities of CAT, SOD, GR and GPOX were higher in cells treated with 60 μM Fe than control and 240 μM Fe. At the highest Fe concentration, the activity of these enzymes tended to be the same or lower than controls. Finally, there was no significant change in GST activity as compared to the control.

Discussion

Fe is important for plants and animals as a nutrient to increase plant productivity and food quality (Briat et al. 2009). Due to its redox properties, Fe participates in many cellular redox reactions, and it is essential for electron transfer chains in the photochemical reactions of photosynthesis and respiration (Briat et al. 1995, 2009). It is also involved in the synthesis of chloroplastic pigments and thylakoid proteins (Kumar et al. 2008a). However, due to its strong reactivity with O_2, Fe concentrations must be tightly regulated in aerobic organisms (Briat et al. 2009).

Plants have developed several mechanisms to maintain a constant concentration of mineral nutrients. Because Fe may cause oxidative damage in the presence of O_2 and H_2O_2, excess Fe must be stored in a safe way within the cell (Caro and Puntarulo 1996). The protein ferritin is an active molecule that incorporates Fe, regulating its cellular availability and preventing potential oxidative damage (Arosio et al. 2009; Lescure et al. 1991). As part of the controlling mechanism, the synthesis of ferritin in plants is responsive to excess Fe (Briat et al. 2009). Fe treatment induced 50- to 60-fold increases in the transcription rate of soybean ferritin mRNA in cell culture, with a corresponding accumulation of the ferritin protein (Lescure et al. 1991). Increased ferritin expression was also reported for maize (Fobis-Loisy et al. 1995) and *A. thaliana* (Gaymard et al. 1996). Similar to studies in other plants, we found that excess Fe resulted in an increase in *CaFER1* expression in suspension cell cultures of *C. arabica* (Fig. 3a).

Ferritin synthesis is not only induced by Fe and signalling molecules such as H_2O_2 and NO, but also by ozone exposure and high light intensity (Briat et al. 2009). Depending on the environmental signal or developmental stages, members of the ferritin family are differentially expressed in *A. thaliana* (Briat et al. 2009; Petit et al. 2001). In maize, the kinetics of accumulation of the transcripts of two ferritin genes changed as plants were exposed to Fe (Fobis-Loisy et al. 1995). *ZmFer1* accumulated rapidly in response to Fe treatment, while *ZmFer2* also accumulated in response to exogenous abscisic acid, indicating that some ferritins could be also

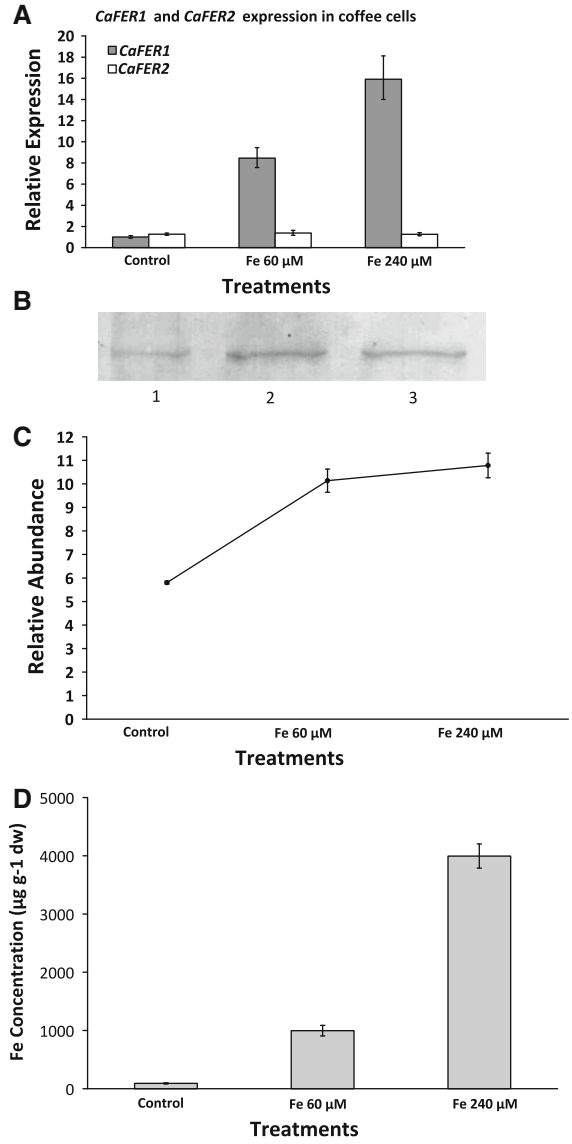

Fig. 3 **a** Quantitative PCR analysis of mRNA from the coffee ferritin genes *CaFer1* and *CaFer2* in coffee cells treated with 60 and 240 µM Fe compared to the constitutively expressed rpl39 gene. Columns represent average values obtained from three independent replicates and vertical bars indicate minimum and maximum relative expression values of three independent replicates. **b** Western blot analysis of *Ca*FER1 protein using a polyclonal anti-ferritin antibody. All reactions were performed using 20 µg of total protein extracts. *Lane 1*—control; *lane 2*—60 µM Fe; *lane 3*—240 µM Fe. **c** Relative abundance of ferritn protein in coffee cells treated with Fe obtained by densitometric analysis. Results expressed as mean and standard error of the mean (±SEM) of three independent replicates. **d** Fe accumulation in cultured coffee cells grown for 72 h in 60 and 240 µM Fe. Values are the means of three replicates ±SEM

involved in other stress responses (Fobis-Loisy et al. 1995). In *A. thaliana*, the ferritin gene *AtFer2* is abundantly expressed in mature siliques and dry seeds (Ravet et al. 2009). *CaFER1* and *CaFER2* showed different patterns of expression in *C. arabica* cells, as exogenous Fe did not induce *CaFER2* transcripts in the conditions used in this study. A search of the libraries of Brazilian Coffee Genome Project (Vieira et al. 2006) showed that *CaFER2* is only weakly expressed in all tissues, with the highest expression in fruits (Nobile et al. 2010). Therefore, it is possible that *CaFER2* is related to Fe accumulation in coffee beans; this hypothesis is currently under investigation.

Phylogenetic analysis revealed that the deduced amino acid sequence of *CaFER1* is similar to a few well-characterised ferritins, with the highest amino acid identity to the ferritins of *P. pyrifolia* (66%), *P. trichocarpa* (67%), *S. tuberosum* (74%), and *G. max Fer*H1 (75%) and *Fer*H2 (72%) (BLAST scores 2e-92, 6e-91, 1e-80, 5e-95, and 2e-89, respectively; Fig. 2). *S. tuberosum* ferritin respond to pathogen attack in leaves and to elicitor (eicosapentaenoic acid) treatment in tubers, suggesting that hydrogen peroxide release, triggered by the pathogen and elicitor, was responsible for increasing ferritin expression (Mata et al. 2001). Similar to *CaFER*1, soybean ferritin mRNA increased 52-fold when cell cultures were incubated with Fe, which is consistent with an increase of ferritin protein (Lescure et al. 1991). The deduced amino acid sequence of *CaFER2* exhibited the highest similarity with ferritins from *N. tabacum* (*Nt*FER1 and *Nt*FER2) and *L. esculentum* (*Le*FER), and it was closer to *At*FER2 than to the other ferritins of *A. thaliana* (Fig. 2). The highest BLAST score of *Ca*FER2 was with *S. tuberosum* (2e-104). Although tomato is a better genomic model for coffee than *A. thaliana* because they both belong to the Asterid I clade of dicot plant family (Lin et al. 2005) and share similar chromosome architectures, ferritin *Ca*FER1 did not show high similarity with ferritin of *L. esculentum* (identities = 62%, BLAST score = 3e-49). On the other hand, the *Ca*FER2 exhibited a 70% identity and a BLAST score of 1e-69 (Fig. 2).

Although in the last few years the literature has been flooded with studies on metal toxicity in plants, little is known about this process in coffee, with the majority of these reports in coffee using cell cultures as a model (Gomes-Junior et al. 2006a, 2006b, 2007; Martínez-Estévez et al. 2001a, b). Among these studies, only three focused on the antioxidant enzyme response to metals (Gomes-Junior et al. 2006a, 2006b, 2007). As far as we are aware, this is the first report on the oxidative effects of Fe in coffee using cell cultures as a model.

In coffee cells, Fe induced an increase in activity of all analysed enzymes, except GST, mainly at 60 μM, indicating that a stressful condition was imposed on the cells, even though lipid peroxidation and ROS production were not determined. However, previous reports with the same coffee cell suspension cultures subjected to Cd, Ni and Se exposure have shown dramatic increases in the rate of lipid peroxidation (Gomes-Junior et al. 2006a, 2006b, 2007), which may likely be the case for Fe toxicity as well. The reduction in activity to near/similar (CAT, GR, GPOX) or below (SOD) control levels observed when Fe was increased further (240 μM) indicates that the response to Fe by the antioxidant system is quick and efficient, being triggered and responding promptly when exposed to low Fe concentrations. It is even possible that the responses by other antioxidant enzymes may vary at different Fe concentrations or during cell growth, as shown recently by Gratão et al. (2008) in cell suspension cultures of tobacco subjected to Cd and Ni. These authors observed that during the early stages of exposure to Cd, GR and GST initially responded to the oxidative stress induced by the metal, whereas CAT and GPOX were active later.

Such a differential response and change in the pattern of response to distinct metal concentrations and times of exposure is critical. For instance, the same reports with coffee cell suspension cultures exposed to Cd, Ni and Se revealed that a new GST isoenzyme was induced only after 24 h of exposure to these metals, indicating an early specific, unique response of one particular isoenzyme (Gomes-Junior et al. 2006a, 2006b, 2007).

The increased expression of *CaFER1* in response to Fe was much higher at 240 μM than at 60 μM, which also suggests a balance between two distinct operating antioxidant systems (i.e., antioxidant enzymes and ferritin) that are coordinately regulated. Thus, at lower Fe concentrations the antioxidant system takes priority, but at much higher concentrations, ferritin takes over as the main component of the protective system.

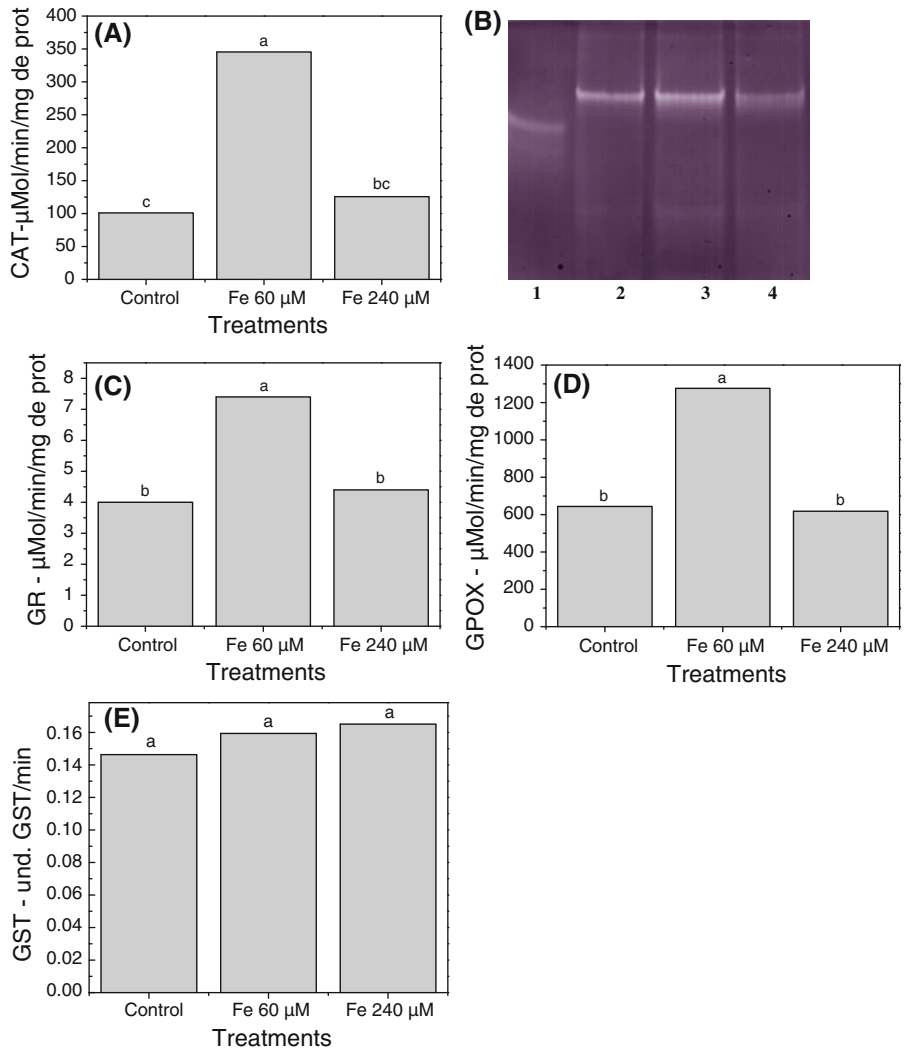

Fig. 4 **a** Specific activity of catalase. **b** Activity staining for superoxide dismutase following native PAGE of protein extracts from cultured coffee cells: *lane 1*—bovine SOD standard; *lane 2*—control (zero Fe); lane 3 and 4—cells grown for 72 h in 60 and 240 µM Fe, respectively. **c** Specific activity of glutathione reductase, **d** guaiacol peroxidase and **e** glutathione *S*-transferase in coffee cells grown for 72 h in 60 and 240 µM Fe. Values are the means of three replicates. Means with the same letter are not significantly different ($P < 0.05$) by Tukey's test

Although data for cell growth are not presented here, the 240 µM Fe concentration 'partially' inhibited cell growth, indicating a more damaging effect and perhaps a more stressful oxidative condition that the tested enzymes were not able to respond to effectively. It is likely that at different concentrations of Fe, distinct groups of enzymes respond to the stress, which is suggested by the lack of response by GST to the Fe-induced stress.

The changes in ferritin expression and enzyme activities shown here, together with the data from previous reports with other metals (Gomes-Junior et al. 2006a, 2006b, 2007), clearly suggest that coffee has an efficient antioxidant response to metal-induced oxidative stress.

In plants, several studies indicate that excess Fe leads to an increase in ROS production and promotes oxidative stress responses. Our results strongly indicate that toxic levels of Fe lead to an increased uptake of Fe in coffee cell suspensions, significantly altering the levels of ferritin mRNA and the activity of some antioxidant enzymes. This indicates that high Fe

concentrations activate a range of cellular responses related to stressful conditions and that Fe availability within the cell must be finely regulated to avoid cellular damage. The next step in these studies is to verify the response of ferritin expression in coffee plants growing in the field upon exposure to Fe and other metals as well as to study how Fe affects coffee fruit physiology.

Acknowledgments The authors are grateful to Dr. Janette Palma Fett (Universidade Federal do Rio Grande do Sul) for providing the polyclonal anti-ferritin antibody used in this study. This work was supported by grants from Fundação de Amparo a Pesquisa do Estado de São Paulo (Fapesp) and Conselho Nacional de Desenvolvimento Científico e Tecnológico Brasil (CNPq).

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