Soybean ferritin expression in *Saccharomyces cerevisiae* modulates iron accumulation and resistance to elevated iron concentrations.

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ABSTRACT

Dietary iron deficiency is a worldwide prevalent nutritional disorder to which women and children are especially vulnerable. A common strategy to combat iron deficiency consists of dietary supplementation with inorganic iron salts, whose bioavailability is low and causes frequently gastric problems. Iron-enriched yeasts and cereals are alternative strategies to diminish iron deficiency. Animals and plants possess large ferritin complexes that accumulate, detoxify or buffer excess cellular iron. However, fungi including yeast *Saccharomyces cerevisiae* lack ferritin and use vacuoles as iron storage organelles. This work explored how plant ferritin expression influenced baker’s yeast iron metabolism. Soybean seed ferritin H1 and H2 (SferH1 and SferH2) genes were cloned and expressed in yeast cells. Both soybean ferritins assembled as multimeric complexes, which bound yeast intracellular iron *in vivo* and, consequently, induced the activation of the genes expressed during iron scarcity. Soybean ferritin protected yeast cells that lacked the Ccc1 vacuolar iron detoxification transporter from toxic iron levels by reducing cellular oxidation, thus allowing growth at high iron concentrations. Interestingly when simultaneously expressed in *ccc1Δ* cells, ferritins SferH1 and SferH2 assembled as heteropolymers, which further increased iron resistance and reduced the oxidative stress produced by excess iron compared to ferritin homopolymer complexes. Finally, soybean ferritin expression led to increased iron accumulation in both wild-type and *ccc1Δ* yeast cells at certain environmental iron concentrations. Hence yeasts that express soybean seed ferritin could be explored as a novel strategy to increase dietary iron absorption.
Iron deficiency is a worldwide nutritional disorder to which women and children are especially vulnerable. A common strategy to combat iron deficiency consists of dietary supplementation with inorganic iron salts, whose bioavailability is very low. Iron-enriched yeasts and cereals are alternative strategies to diminish iron deficiency. Animals and plants possess large ferritin complexes that accumulate, detoxify or buffer excess cellular iron. However, the yeast *Saccharomyces cerevisiae* lacks ferritin and uses vacuoles as iron storage organelles. Here, we explored how soybean ferritin expression influenced yeast iron metabolism. We observed that soybean ferritin bound yeast intracellular iron, protected iron-sensitive yeast cells from toxic iron levels by reducing cellular oxidation, and allowed growth at high iron concentrations. Finally, soybean ferritin expression led to an increase in iron accumulation at certain environmental iron concentrations. Hence yeasts that express soybean seed ferritin could be explored as a novel strategy to increase dietary iron absorption.
Iron is an essential micronutrient for most living organisms because it participates as a redox cofactor in many metabolic pathways, including respiration, lipid biosynthesis, translation, DNA replication and photosynthesis. Despite its abundance, iron bioavailability is very low because Fe\(^{3+}\) forms ferric hydroxides that tend to precipitate at a physiological pH. In humans, iron deficiency anemia (IDA) is the most extended and common nutritional disorder worldwide. The World Health Organization estimates that IDA affects approximately one fourth of the world population, with especial incidence in women and children. Consequences of IDA include diminished learning ability in infants, fatigue and reduced physical capacity and work productivity in adults, and poor pregnancy outcomes (reviewed in (1,2)). Iron supplementation with ferrous salts is one of the most widely applied strategies to combat IDA. However, such treatment can cause gastric problems like vomiting, faintness, constipation or diarrhea. Furthermore, inorganic iron induces oxidative changes and its absorption in the intestine is vastly altered by diet composition. Many studies have demonstrated that the iron contained in ferritin is soluble and bioavailable to alleviate iron deficiency (3-5). Thus an emerging strategy to fight IDA consists of supplementing iron in the form of ferritin, which protects iron from chelators like the phytates and polyphenols present in the diet, reduces oxidative damage and the risk of gastric problems, and its iron is properly absorbed via receptor-mediated endocytosis (reviewed in (6-10)).
Ferritin is a multimeric iron storage and detoxification protein that is ubiquitously distributed in animals, plants and bacteria, but is absent in fungi. Ferritin complexes consist of 24-subunit heteropolymers that assemble as spherical shells and can store up to 4500 Fe$^{3+}$ ions each (reviewed in (11,12)). In vertebrates, ferritin localizes to the cytoplasm and is composed of two distinct subunits, H and L, whose ratio varies depending on the tissue. The H subunit contains ferroxidase centers, whereas the L subunit facilitates nucleation of the mineral core. Some studies have shown that human ferritin H is also found in cell nuclei (13). The human ferritin encoded in the nuclear genome, expressed as a ferritin H-like precursor, is targeted to mitochondria and processed to a functional subunit that assembles into typical ferritin shells with ferroxidase activity (14).

Despite animal and plant ferritins having evolved from a common ancestor and possessing a conserved primary structure (15), plant ferritins (phytoferritins) exhibit distinctive features, but with some parallels to human mitochondrial ferritin (reviewed in (8,9,16,17)). Phytoferritins localize to non-green plastids and only possess H-type subunits with both a ferroxidase center and a nucleation site. In addition to a four-helix bundle (helices A, B, C and D) and a fifth short helix (helix E), at their amino-terminus, plant ferritin subunits contain a transit peptide (TP) that is responsible for their translocation to the plastid, and a specific domain, known as extension peptide (EP) ((18,19); see Figure 1A). Plant ferritin EP, which localizes to the exterior protein surface, helps stabilize the oligomer and acts as a second ferroxidase center for iron binding and oxidation (20-24). In vitro studies have suggested that SFerH1 EP displays serine protease-like activity that catalyzes its autodegradation by facilitating iron release from ferritin during seed germination (25). Studies in Arabidopsis have indicated that phytoferritin mainly
functions in the buffering of iron needed for metabolic processes and in protecting cells against oxidative stress (26). However in pea seeds, more than 90% of iron is stored in phytoferritin, which is required for plant germination and early growth (27,28). *In vitro* work has shown that soybean seed ferritin H1 and H2 subunits (SFerH1 and SFerH2, respectively) synergistically interact during iron mineralization by exhibiting greater iron oxidation activity than separate homopolymers (29,30). Soybean ferritin has been used to enrich cereals with iron (31-33). Nonetheless, successful plant iron biofortification requires a complex combinatorial approach that simultaneously modifies iron acquisition, transport and accumulation in its edible part (34,35).

Yeast *Saccharomyces cerevisiae* is used as a model organism to decipher the mechanisms that regulate the response of eukaryotic organisms to changes in iron bioavailability. In response to iron limitation, the yeast *Aft1* transcription factor coordinately activates the expression of a set of genes, collectively known as iron regulon, that function in inorganic iron uptake, siderophore-iron acquisition and the remodeling of iron-dependent metabolism (36-38). Iron regulon members include: cupro-ferroxidase *Fet3*, required for high-affinity iron uptake (39); cell wall-associated mannoprotein *Fit3*, involved in siderophore iron transport (40); RNA-binding protein *Cth2*, which rearranges cellular iron metabolism to optimize iron utilization (41). Elevated iron concentrations accelerate the formation of reactive oxygen species (ROS) via Fenton redox reactions, which leads to oxidative damage in cellular membranes, proteins and nucleic acids. In response to high iron concentrations, yeast cells activate the expression of *Ccc1*, a protein that protects cells from iron toxicity by transporting iron from the cytoplasm to the vacuole,
where it is stored bound to polyphosphates (42-44). Thus \( ccc1 \Delta \) yeast strains are sensitive to elevated iron concentrations due to detoxification defects (42).

Baker’s yeast \( S. \textit{cerevisiae} \) is a GRAS (generally recognized as safe) organism used to obtain multiple fermented foods and beverages, livestock feed for fish and poultry and food supplement for human consumption, because it is rich in vital amino acids, fiber and B-type vitamins. Iron-enriched yeasts are currently being evaluated, and appear as promising iron sources to prevent and palliate iron deficiency in humans and animals (45). In fact, recent reports have indicated that iron-enriched baker’s yeasts efficiently help animals recover from iron deficiency, while maintaining their fermentative and bakery properties (46, 47). Previous studies have reported that the expression of animal or human ferritin genes in yeast cells leads to increased iron accumulation (48-50). In this report, we characterized how yeast cells that express two phytoferritin genes, SFerH1 and SFerH2, behave in response to elevated iron concentrations with regard to gene expression, iron accumulation, iron resistance and the cellular redox state.
MATERIALS AND METHODS

Yeast strains and growth conditions. In this study, wild-type BY4741 (MATα, his3Δ1, leu2Δ0, ura3Δ0, met15Δ0) and ccc1Δ (BY4741 ccc1::KanMX) S. cerevisiae strains were used. Yeasts were cultivated at 30°C in synthetic complete SC medium that lacked uracil (SC-ura) or histidine (SC-his), or lacked both (SC-ura-his). Iron was added to autoclaved SC media at the desired final concentration from either 250 mM FeCl₃ (ferric chloride, Sigma) or 100 mM Fe(NH₄)₂(SO₄)₂ (ferrous ammonium sulfate, FAS, Sigma) stock solution. Both solutions were prepared in 0.1M HCl to facilitate iron solubility and were used fresh. A control with the equivalent HCl concentration was always prepared, but did not alter growth. To cultivate yeast cells in liquid media, overnight SC precultures were re-inoculated at an absorbance at 600 nm (A₆₀₀) of 0.2, and then incubated for 24 h at 30°C and 190 rpm in 50-mL flasks that contained 5 mL of SC-ura-his supplemented with the corresponding iron salt concentration (51). Depending on the experiment, these cultures were employed to determine A₆₀₀, cell concentration, dry weight (DW) and endogenous iron. For growth in 96-well plates, exponentially growing yeast cells were re-inoculated at an A₆₀₀ of 0.2 in 260 μl of liquid SC-ura-his medium, and A₆₀₀ was determined in a SPECTROstar Nano microplate reader (BMG Labtech) every 30 min for 3 days at 28°C. The doubling time in the exponential phase was determined. To perform growth assays in solid media, the cells from the overnight precultures were diluted at an A₆₀₀ of 0.1 and then spotted directly, and after 1:10 and 1:100 dilutions in sterile water. For the oxidative stress experiments, methylene blue (Sigma) was added to SC-ura-his or SC-ura-his with FeCl₃ plates from a 1% stock solution. Plates were incubated at 30°C for 3 days and then...
photographed. For Prussian blue staining, yeast transformants were cultivated for 8 h in SC-ura-his with 5 mM ferric citrate to the exponential phase.

Plasmids. pSFeH1 and pAT-T7-SFeH2 plasmids (kindly provided by Dr. Taro Masuda, (29)), which contained soybean (Glycine max) seed ferritin H1 and H2 cDNA, respectively, were used as templates to PCR-amplify the coding sequences of ferritin H1 and H2, which lacked their transit peptide (amino acids 1 to 48 in SFerH1, and amino acids 1 to 47 in SFerH2; see Figure 1A). Oligonucleotides SSFH1-BamHI-F and SSFH1-SalI-R were used to amplify ferritin H1, whereas oligonucleotides SSFH2-BamHI-F and SSFH2-SalI-R were used to obtain ferritin H2 (Table 1). The SFerH1 PCR product was cloned into centromeric vectors p416ADH, p416TEF and p416GPD, and SFerH2 was cloned into centromeric vectors p413ADH, p413TEF and p413GPD (52). The URA3 selection marker was used for SFerH1 plasmids and HIS3 for SFerH2. In all cases, restriction sites BamHI and SalI were utilized for cloning. All the PCR amplifications were performed with Phusion high-fidelity DNA polymerase (Thermo Scientific) and cloned inserts were sequenced. The One Shot TOP10 Escherichia coli strain (Invitrogen) was used for plasmid propagation and isolation.

Western blot analyses. Total protein extracts were obtained by the alkali method (53). Equal amounts of protein were resolved in SDS-PAGE gels and transferred to nitrocellulose membranes. Ponceau staining was used to assess protein transfer. Both the soybean ferritin H1 and H2 protein levels were determined with an anti-
soybean ferritin H1 primary antibody (kindly provided by Dr. Taro Masuda), whereas the Pgk1 protein levels were determined with an anti-Pgk1 (22C5D8, Invitrogen) antibody. Immunoblots were developed with HRP-labeled secondary antibodies and the ECL Select Western Blotting Detection Kit (GE Healthcare Life Sciences). Immunoblot images were obtained with an ImageQuant LAS 4000 mini Biomolecular Imager (GE Healthcare Life Sciences).

Prussian blue staining. To determine whether the soybean ferritin expressed in yeast assembled into the multimeric complexes that stored iron, staining with Prussian blue was performed as previously described (49). Briefly, yeast cells that corresponded to a total optical density at 600 nm (OD600) of 60 were harvested, and washed twice with distilled water and once with extraction buffer (20 mM Tris-HCl pH 7.4). Then cells were disrupted in 200 μL of extraction buffer and 200 μL of glass beads in a bead beater (Biospec Products Inc.). Cell lysates were centrifuged 3 min at 10000 g at 4°C and the supernatant was transferred to a fresh tube. Since ferritin is a thermostable protein, it was partially purified from yeast cell extracts by heating at 75°C for 15 min. Cell extracts were centrifuged 10 min at 16000 g, the supernatant was transferred to a fresh tube and protein concentrations were determined by the Bradford method. Ten μg of proteins were resolved by 7% native PAGE under non denaturing conditions. Two identical gels were prepared: one was stained for protein using 0.2% Coomassie brilliant blue, and the other was stained for iron loading with a fresh 1:1 (v:v) mixture of 2% K₄Fe(CN)₆ (Ferrocyanide or Prussian blue, Sigma) and 2% 11.6 M HCl (0.116 M...
final HCl concentration). Horse spleen ferritin (Sigma) was included as a control (data not shown).

Endogenous iron measurements. Yeast cells that corresponded to a total OD$_{600}$ of 5.0 were collected by centrifugation, washed twice with 1 mM EDTA and once with milli-Q water. Cells were dissolved in 500 μL of 3% HNO$_3$ and incubated at 98ºC for 16 hours with agitation. After digestion, extracts were used to determine iron levels. For the yeast cells grown in SC, iron levels were determined by ICP-MS at the Servei Central d'Instrumentació Científica of the Universitat Jaume I (Castellón, Spain). We used a colorimetric assay for the other samples (54). We previously confirmed that both methods provided similar results (51).

RNA analyses. To determine the expression levels of the specific mRNAs, total RNA was extracted and quantitative reverse transcription PCR (qPCR) was performed as previously described (55). For each analyzed gene, a standard curve was made with serial dilutions of the cDNA sample. The ACT1 mRNA values were used to normalize data. Data and error bars represented the average and standard deviation of at least three independent biological samples. All the primers used in this study are listed in Table 1.

Cell concentration and yeast dry weight. Yeast cells incubated in liquid cultures were properly diluted and sonicated before their concentration (cells/mL) was determined in a Beckman Coulter Counter Z. To establish yeast DW, the cells that
corresponded to a total OD$_{600}$ of 30 were collected by centrifugation and washed with distilled water. Then they were transferred to a previously weighed Eppendorf tube and dried at 50°C for 3 days. Finally, yeast DW was obtained by subtracting the total weight from the Eppendorf tube weight. At least three independent biological samples were performed to determine the yeast cellular concentration and DW.

**Statistical analyses.** To evaluate statistical significance, the Figure 5B data were analyzed by the multiple comparison Tukey test using the SigmaPlot 12.5 software. In this case, the endogenous iron levels of the $ccc1\Delta$ strain transformed with soybean ferritins (SFerH1, SFerH2, SFerH1 + SFerH2) were compared to the iron content in the $ccc1\Delta$ cells that lacked ferritin (vector) grown in SC with 10 μM or 100 μM FAS. The statistical significance of the endogenous iron levels of the wild-type strains in Figure 6B was determined by a two-way analysis of variance (ANOVA) using the GraphPad Prism 6 software. The ferritin and iron concentrations in the growth medium were considered the ANOVA variables. In all cases, the $P$ value was < 0.006.
RESULTS

Cloning and expression of soybean seed ferritin in *Saccharomyces cerevisiae*.

Unlike mammalian ferritins, which reside in the cytoplasm, phytoferritins localize to plastids. Therefore in order to express seed SFerH1 and SFerH2 in baker’s yeast, both ferritin-coding sequences lacking their transit peptide were cloned into the vectors that drove expression under the control of yeast gene promoters *ADH1, TEF2* or *TDH3* (Figure 1A; see Materials and Methods for more details). The wild-type cells that expressed soybean ferritins were grown in synthetic media (SC-ura for SFerH1 or SC-his for SFerH2) to the exponential phase and proteins were extracted. To determine ferritin levels, a Western blot was performed with an anti-SFerH1 antibody, which cross-reacted with SFerH2. As expected, the maximal expression in both cases was achieved for the GPD plasmids that drove gene expression with *TDH3* (the strongest expression promoter) and minimum ferritin levels were obtained for *ADH1* (the weakest promoter) (Figure 1B). Similar expression results were observed when yeast cells were incubated in the presence of iron in growth medium or for longer time periods (data not shown). Surprisingly, the expression in the multicopy 2μ plasmids did not lead to higher ferritin levels (data not shown). Thereafter SFerH1 and SFerH2 genes were expressed in the centromeric plasmids under the control of the constitutive *TDH3 (GPD)* promoter.

Soybean ferritin binds iron when expressed in yeast.
To address whether the soybean ferritin expressed in yeast assembled into functional complexes was capable of capturing intracellular iron, Prussian blue staining was performed, which detects the iron bound to ferritin (49). The wild-type cells that lacked ferritin, and which expressed either SFeH1, SFeH2, or both genes, were simultaneously grown to the exponential phase in medium with 5 mM ferric citrate. Proteins were extracted, resolved by PAGE under non-denaturing conditions and stained with ferrocyanide (Prussian blue). Characteristic blue bands were observed in the cells that expressed SFeH1 or SFeH2, which was consistent with the multimeric ferritin complexes that bound iron in vivo (Figure 2A). Prussian blue staining dramatically decreased when the yeast cells that expressed ferritin were grown in media with no added iron (data not shown). As expected, SFeH2 assembled into a complex larger than SFeH1, whereas a heteropolimer of an intermediate size formed when both SFeH1 and SFeH2 were co-expressed in the same cells. A similar result was obtained for the ccc1Δ mutant cells (data not shown). These results demonstrated that the soybean seed ferritin expressed in yeast assembled into a multimeric complex that bound iron in vivo.

To further address how soybean ferritin influenced iron homeostasis when expressed in yeast, the wild-type cells transformed with vector SFeH1 or SFeH2, or both genes, were grown, and the expression of specific iron-regulated genes, such as CTH2, FET3 and FIT3, was determined by qPCR. As shown in Figure 2B, the expression of all these three genes increased in ferritin-expressing cells, especially in the SFeH1 cells. When the experiment was performed in the cells that lacked the Ccc1 vacuolar iron transporter, both SFeH1 and SFeH2 also brought about an increase in the CTH2, FET3 and FIT3 mRNA levels. Interestingly, SFeH1 and
SFeH2 co-expression in ccc1Δ cells caused a further induction of these iron-regulated genes. Since CTH2, FET3 and FIT3 genes are members of the iron regulon, which are up-regulated by the Aft1 transcription factor when cells sense iron deficiency, these results indicated that soybean ferritin reduced intracellular iron availability. Taken together, our results strongly suggested that the soybean ferritin expressed in yeast bound iron in vivo, and cells perceived it by responding accordingly.

Soybean ferritin increases ccc1Δ resistance to elevated iron concentrations.

As soybean seed ferritin bound iron when expressed in yeast cells, a decision was made to determine whether ferritin conferred resistance to toxic levels of extracellular iron. For this purpose, wild-type and ccc1Δ cells were transformed with the plasmids that expressed SFerH1 and SFerH2, and growth on solid media that contained increasing FeCl₃ concentrations was assayed. Spot assays were performed, which consisted in 10-fold dilutions of yeast drops starting at an A₆₀₀ of 0.1 on SC-ura-his plates, to maintain the selection for both SFerH1 and SFerH2 plasmids, or for empty vectors. When expressed in wild-type cells, soybean ferritin did not alter growth on the iron-containing plates (data not shown). However, the ccc1Δ cells that expressed either SFerH1 or SFerH2 grew better at 4 mM FeCl₃ than the ccc1Δ cells without ferritin (Figure 3A). SFerH1 and SFerH2 allowed growth at 5 mM FeCl₃, an inhibitory concentration for the cells that lacked the Ccc1 detoxifying factor (Figure 3A). No growth was observed at higher FeCl₃ concentrations (data not shown). To further characterize the effect produced by soybean ferritin to ccc1Δ iron resistance, growth in 96-well plates
with synthetic liquid medium that contained 5 mM of another iron salt, ferrous ammonium sulfate (FAS), was assayed. Soybean ferritin expression did not alter yeast growth in liquid SC-ura-his (Figure 3B). It was noteworthy that the expression of either SFerH1 or SFerH2, either separately or simultaneously, markedly increased the growth rate when 5 mM FAS was added to the medium (Figure 3C). The co-expression of SFerH1 and SFerH2 yielded maximal growth at high iron concentrations (Figure 3C). Similar conclusions were drawn after calculating the doubling time in the exponential growth phase. Whereas no difference was observed in synthetic medium, the doubling time was shorter when cells were grown in 5 mM FAS (Figure 3D). The yeast cells that lacked ferritin obtained the longest doubling time, whereas the yeasts that co-expressed both phytoferritins provided the most rapid growth in the exponential phase (Figure 3D). These results indicated that soybean ferritin increased ccc1Δ resistance to high iron by probably detoxifying intracellular iron. An additive effect was observed when both H1 and H2 phytoferritin forms were co-expressed.

Soybean ferritin reduces yeast oxidative stress produced by elevated iron concentrations.

Iron at high concentrations is harmful to cells because it catalyzes the redox reactions that generate ROS. As soybean ferritin captures iron in yeast, a decision was made to determine the cellular redox state in the ccc1Δ cells that expressed soybean seed ferritin compared to the control ccc1Δ yeasts. For this purpose, growth on the plates that contained biocompatible redox indicator methylene blue was assayed, which gains a blue color when oxidized (56). As observed in Figure 4,
the expressions of SFerH1 and SFerH2 separately, but especially the co-expression of both phytoferritins, markedly reduced the blue color induced by increasing FeCl$_3$ concentrations. This indicated that soybean ferritin reduced the cellular redox state. Therefore, these results suggested that soybean ferritin expression protected yeast cells from the oxidative stress produced by excess iron.

**Soybean ferritin expression increases yeast iron accumulation.**

We herein showed that soybean seed ferritin expressed in yeast bound iron *in vivo* increased *ccc1Δ* resistance to high iron concentrations and reduced the redox state of the *ccc1Δ* cells grown at toxic iron levels. Therefore, whether soybean ferritin expression allowed yeast cells to increase iron accumulation was ascertained. As shown in Figure 5A, the *ccc1Δ* cells that expressed soybean ferritin reached a slightly higher maximal cell density than the cells that lacked *CCC1* when incubated in synthetic medium with no additional iron. Maximal cell density did not alter when 10 μM or 100 μM FAS were added to the growth medium. However, yeast growth was negatively affected at 1 mM or at higher FAS concentrations (Figure 5A). Consistently with Figure 3, soybean ferritin expression increased *ccc1Δ* resistance to high iron concentrations. Whereas the *ccc1Δ* strain only grew up to 3 mM FAS, the *ccc1Δ* cells that expressed either SFerH1 or SFerH2 were able to grow at 6 mM FAS, and the *ccc1Δ* cells that co-expressed both phytoferritin forms reached 8 mM FAS (Figure 5A).

In order to determine yeast cellular iron concentrations as weight of iron per yeast DW (g Fe/g DW), the endogenous iron levels and the yeast DW of each
transformant were measured after 1 day of incubation in each tested iron-containing medium (Materials and Methods; (51)). No significant differences were found in the iron accumulation by the $ccc1\Delta$ cells incubated in synthetic medium with no additional iron (Figure 5B). Although growth was not affected, addition of 10 $\mu$M or 100 $\mu$M FAS to synthetic medium allowed the $ccc1\Delta$ ferritin-expressing cells to significantly increase iron accumulation (Figure 5B). Iron levels were maximal for the SFerH1/SFerH2 co-expressing cells, which reached 3-fold higher endogenous iron levels than the $ccc1\Delta$ cells that lacked ferritin when incubated at 0.1 mM FAS (around 400 $\mu$g Fe/g DW; Figure 5B). Yeast cells gradually increased iron content as the environmental FAS concentration rose (Figures 5C). It was noteworthy that the $ccc1\Delta$ cells which expressed ferritin did not accumulate more iron than the control cells at 1 and 3 mM FAS (Figure 5C). The yeast $ccc1\Delta$ cells that expressed soybean ferritin reached maximal endogenous iron levels (around 7 mg Fe/g DW) when incubated in 6 or 8 mM FAS in the medium (Figure 5C).

Although no iron resistance alteration took place when the wild-type cells that expressed soybean ferritin were assayed for growth on the solid media with increasing iron concentrations (data not shown), the results obtained for the $ccc1\Delta$ cells in liquid conditions encouraged further characterizing the effects of soybean ferritin expression in wild-type cells. Similarly to $ccc1\Delta$, the wild-type cells transformed with empty vectors SFerH1 or SFerH2, or with both phytoferritin forms, were cultivated for 1 day in iron-containing media, and both cell density and endogenous iron levels were determined. As expected, wild-type cells were more resistant to iron than $ccc1\Delta$ cells, even when grown in the media with 8 mM FAS (Figure 6A), whereas $ccc1\Delta$ cells only tolerated up to 3 mM FAS (Figure 5A).
Soybean ferritin expression did not alter wild-type cell growth. The only exception was the 8 mM FAS-containing medium, which caused slightly reduced growth (Figure 6A). Soybean ferritins significantly increased iron accumulation (up to 26%) in the wild-type cells grown in the media with 1 mM or at higher iron concentrations (Figure 6B). Taken together, these results suggested that at certain environmental iron concentrations, soybean seed ferritin expression facilitates iron accumulation in both wild-type and ccc1Δyeast cells.

DISCUSSION

In this report we explored how soybean seed ferritin expression influenced yeast iron metabolism. By using Prussian blue staining, the fact that soybean seed ferritin bound iron in vivo when expressed in yeast cells was first demonstrated. Interestingly, iron accumulation in phytoferritin complexes led to the transcriptional activation of Aft1 target genes probably due to a reduction of intracellular iron availability for iron-sulfur cluster biosynthesis. The induction of iron-responsive genes has also been observed when human ferritin was expressed in yeast cells (57).

Plant studies have shown that EP-mediated ferritin autodegradation facilitates iron release from soybean ferritin and its utilization during seed germination (25). Although yeast-expressed phytoferritin stores iron in a non-toxic state, it is unclear whether iron bound to soybean ferritin can be recycled to be utilized in yeast metabolism. Previous studies have demonstrated that human mitochondrial ferritin protects yeast frataxin mutants from death induced by
oxidative stress and excess iron or copper, extends lifespan and improves respiratory function (58-60). However, human mitochondrial ferritin is not a functional homolog of yeast frataxin (60). Instead, the yeast expression of human ferritin makes mitochondrial iron more available for heme and cytochrome synthesis in mutants that accumulate insoluble and biologically unavailable iron in mitochondria, such as those with impaired Fe-S cluster biogenesis (60,61). Although the mechanism is not known, it has been proposed that ferritin can bind a small proportion of mitochondrial precipitated iron and release it as Fe\(^{2+}\) to restore heme synthesis (60).

In animals, excess iron is stored in the form of ferritin, whereas plant cells primarily keep iron supplies in vacuoles. Arabidopsis studies have suggested that phytoferritin functions in iron buffering by protecting cells against oxidative stress (26). However, most iron in pea seeds is stored in the form of phytoferritin, which is critical for germination and early growth (27,28). Here it was observed that soybean ferritin exerted both iron storage and oxidative protection functions when expressed in yeast. First, soybean ferritin expression increased yeast iron accumulation; second, ferritin enhanced yeast resistance to elevated iron concentrations; third, ferritin reduced the yeast redox state under high-iron conditions. All these observations became even more evident in ccc1Δ mutants than in wild-type cells, probably because the mutant strain lacked the vacuolar iron-detoxification pathway, which protects wild-type cells from excess iron and partially masks the ferritin effect. The present work actually showed that soybean ferritins protected ccc1Δ yeast cells from iron toxicity, despite previous studies that used human ferritins not being able to show a protective effect when
expressed in a $ccc1\Delta$ strain (62). Previous *in vitro* studies have reported that
SFeR1/SFeR2 heteropolymers display greater iron oxidation activity and DNA
protection from oxidative damage during iron oxidative deposition than soybean
ferritin homopolymers (30,63). Here it was observed that simultaneously
expressed SFeR1 and SFeR2 assembled into heteropolymers in yeast. Moreover,
heteropolymers displayed greater iron accumulation and resistance, and lower
oxidative stress than ferritin homopolymers when expressed in $ccc1\Delta$ yeast cells.
This finding indicated that ferritins H1 and H2 positively interacted in yeast.

Plant iron biofortification seems the most appropriate strategy to combat
IDA worldwide (64). Biotechnological approaches, which have consisted in
soybean seed ferritin ectopic expression in cereals, have only moderately enriched
iron content (31-33). Since plants are multicellular organisms, genetic engineering
strategies need to improve various aspects of iron homeostasis, including root iron
uptake, transport to edible parts, resistance to excess iron and bioavailability, in
order to obtain appropriate fortified vegetable foods (34,35). By using a unicellular
organism such as *S. cerevisiae*, the present work bypassed several obstacles.
Soybean seed ferritin up-regulated the expression of yeast iron uptake machinery,
which probably contributed to iron accumulation. Previous studies with human
ferritins have reported increases in yeast endogenous iron accumulation, which
ranged from 2- to 5-fold when cells were incubated with 20 mM ferric citrate (49),
However, our study obtained a modest iron increase when wild-type cells were
grown in 8 mM FAS (Figure 6B), as well as a 3-fold increase for the $ccc1\Delta$ cells
grown in 100 μM FAS (Figure 5B). In our case, the main problem that limited iron
accumulation was that soybean ferritin expression levels were too low. Since it
was not possible to increase ferritin expression by using multicopy plasmids, additional approaches need to be explored. For instance, previous studies have substantially enhanced the expression of animal or human ferritin in yeast by using specific yeast strains defective in vacuolar proteases Pep4 and Prb1, by modifying codon translation efficiency in the amino-terminal region, and by optimizing incubation times and iron concentrations (48-50). Additional approaches could include the use of yeast for the high-throughput genetic selection of engineered phytoferritin variants with enhanced iron accumulation capacity, which has been recently done with bacterial ferritin (65); or by identifying and expressing in yeast a phytoferritin chaperone that increases iron loading, which has been described for human PCBP1 ferritin chaperone (66).

In summary, this report demonstrates that plant ferritins assemble into functional complexes that capture iron and protect yeast cells from the oxidative stress derived from high environmental iron concentrations. Although further work is necessary to improve soybean ferritin expression and, consequently, iron accumulation in yeast, this approach represents a promising novel dietary iron source to treat iron deficiency.

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Figure 1. Soybean seed ferritin expression in *Saccharomyces cerevisiae*. (A) Schematic representation of most relevant soybean seed ferritin H1 (SFerH1) and H2 (SFerH2) domains. Numbers indicate amino acid position. TP: transit peptide; EP: extension peptide. (B) Soybean ferritin levels obtained with different yeast expression vectors. Wild-type BY4741 yeast cells transformed with plasmids p416ADH-SFerH1, p416TEF-SFerH1, p416GPD-SFerH1, p413ADH-SFerH2, p413TEF-SFerH2, or p413GPD-SFerH2 were cultivated in SC-ura (SFerH1) or SC-his (SFerH2) to the exponential growth phase, total protein was extracted, and soybean seed ferritin levels were determined by Western blot with an anti-SFerH1 antibody. Pgk1 protein levels were used as a loading control.

Figure 2. Soybean ferritin accumulates iron when expressed in yeast. Wild-type BY4741 and *ccc1Δ* yeast strains were cotransformed with p416GPD + p413GPD (Vector), p416GPD-SFerH1 + p413GPD (SFerH1), p416GPD + p413GPD-SFerH2, or p416GPD-SFerH1 + p413GPD-SFerH2 (SFerH1 + SFerH2). (A) Prussian blue staining of soybean seed ferritin expressed in wild-type yeast cells. Yeast transformants were grown in SC-ura-his with 5 mM ferric citrate for 8 h to the exponential phase. Heat-labile proteins were removed from cell extracts via a 15-minute heating treatment at 75°C. Protein samples were resolved in two 7% non-denaturing polyacrilamide gels. Samples were stained for protein with Coomassie blue and for iron with Prussian blue. (B) and (C) mRNA levels of iron-
regulated genes in the wild-type (B) or ccc1Δ (C) cells that expressed soybean seed ferritin. Yeast transformants were cultivated to the exponential growth phase in SC-ura-his without additional iron, total RNA was extracted and the CTH2, FET3 and FIT3 mRNA levels were determined by qPCR. ACT1 was used to normalize the mRNA values. The average and standard deviation of at least three independent biological experiments is represented.

Figure 3. Growth of the ccc1Δ strain that expresses soybean ferritin in media with high iron concentrations. Yeast ccc1Δ cells were transformed with the plasmids that expressed soybean seed ferritin detailed in Figure 2. (A) After overnight growth in SC-ura-his medium, yeast cells were spotted on 1:10 dilutions starting at A600 = 0.1 on SC-ura-his solid plates that contained increasing FeCl3 concentrations. Plates were incubated at 30°C for 3 days and then photographed. (B) and (C) ccc1Δ transformants were grown to the exponential phase in SC-ura-his and then re-inoculated in 96-well plates at A600 = 0.2 in liquid SC-ura-his without (B) or with (C) 5 mM Fe(NH4)2(SO4)2 (FAS). Cells were incubated for 3 days at 28°C and A600 was determined every 30 minutes with a SPECTROstar Nano microplate reader. The ccc1Δ yeast strains transformed with p416GPD + p413GPD (vector) are shown in blue, p416GPD-SFerH1 + p413GPD (SFerH1) in red, p416GPD + p413GPD-SFerH2 (SFerH2) in green, and p416GPD-SFerH1 + p413GPD-SFerH2 (SFerH1 + SFerH2) in purple. A representative experiment from at least three independent biological replicates is shown in panels B and C. (D) Doubling time in the exponential phase of the growth curves shown in panels B
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**Figure 4. Growth of the ccc1Δ yeasts that expresses soybean ferritin on the plates containing methylene blue redox state indicator.** Yeast ccc1Δ cells were transformed with the plasmids that expressed soybean seed ferritin detailed in Figure 2 and then spotted as indicated in Figure 3A. Methylene blue was added at 1 mM to SC-ura-his plates with or without the indicated FeCl₃ concentrations. Plates were incubated at 30°C for 3 days and then photographed.

**Figure 5. Iron accumulation in the ccc1Δ cells that express soybean ferritin.** Yeast ccc1Δ cells were transformed with the plasmids that expressed soybean seed ferritin detailed in Figure 2. Then yeast transformants were inoculated at A₆₀₀ = 0.2 in SC-ura-his (SC) alone or with increasing Fe(NH₄)₂(SO₄)₂ (FAS) concentrations. Number of cells per mL (A) and endogenous iron content displayed as weight of iron per yeast dry weight (B and C) were determined after 24 h of incubation at 30°C. Data and error bars represent the average and standard deviation of at least three independent biological samples. Asterisks indicate statistically significant differences compared to the ccc1Δ cells that lacked soybean ferritin (P<0.01).

**Figure 6. Iron accumulation in the wild-type cells that express soybean ferritin.** Wild-type BY4741 yeast cells were transformed with the plasmids that expressed soybean seed ferritin as detailed in Figure 2. Then yeast cells were
inoculated at $A_{600} = 0.2$ in SC-ura-his (SC) alone or with increasing Fe(NH$_4$)$_2$(SO$_4$)$_2$ (FAS) concentrations. Number of cells per mL (A) and endogenous iron content displayed as weight of iron per yeast dry weight (B) were determined after 24 h of incubation at 30ºC. Data and error bars represent the average and standard deviation of at least three independent biological samples. Asterisks indicate statistically significant differences compared to the wild-type cells that lacked soybean ferritin ($P<0.006$).

**Table 1. The oligonucleotides used in this work.**

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