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Post-transcriptional regulation of plant ferritin accumulation in response to iron as observed in the maize mutant *ys1*

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Abstract The maize mutant *ys1* accumulates iron in leaves to a lower extent than a Fe-efficient genotype. In this mutant, ferritin mRNA accumulates in response to iron to a similar level as in other genotypes. However, ferritin protein and mRNA abundance does not correlate in *ys1* leaves, demonstrating that iron also controls plant ferritin protein accumulation at the post-transcriptional level.

Key words: Plant ferritin; Gene regulation; Post-transcription; Iron; *Zea mays*

1. Introduction

Regulation of gene expression in eukaryotes in response to variations in cellular metal concentrations occurs both at the transcriptional and at the post-transcriptional level [1–3]. Among various model systems, the iron-regulated synthesis of the iron storage protein ferritin and of the transferrin receptor (TfR), which is responsible for iron uptake, has been widely used to elucidate an integrated pathway acting at the post-transcriptional level in animal cells [2]. Iron responsive elements (IREs) have been found to be stem loops located within the 3′ untranslated region of the TfR mRNA and within the 5′ untranslated region of the ferritin mRNA [4–6]. At least two related iron regulatory proteins (IRP1 and IRP2) are able to bind to IREs of both TfR and ferritin mRNAs [7–9]. IRP1 is shown to have sequence homology with mitochondrial aconitase and furthermore contains a 4Fe-4S cluster important for iron-dependent regulation. Indeed, analysis of recombinant IRP1 led to the observation that the protein functioned as a cytosolic aconitase in iron-replete cells, whereas it functioned as a high affinity IRE-binding protein in iron-depleted cells [10]. When cellular iron levels are low, IRPs bind to IREs, and thereby inhibit translation (ferritin) or mRNA degradation (TfR). In plants, 5′ untranslated regions of ferritin mRNA do not contain IRE, nor is it found in known soybean and maize ferritin genes [11–16]. In addition, although an aconitase gene of *Arabidopsis thaliana* has been cloned recently which encodes a protein with significant homology with mammalian IRP1 [17], no IRP activity, as examined by RNA gel-shift mobility assays using an animal IRE as a probe, has been detected in yeast and plant extracts [18]. Consistent with these observations, animal ferritin mRNAs are translated perfectly well in an in vitro wheat germ system, while their translation is repressed in a rabbit reticulocyte system which contains endogenous IRPs; this translational repression can be conferred on the plant translation system by biochemical complementation with purified IRP1 from rabbit liver [19]. Indeed, the pioneering work of Van der Mark et al. [20] strongly suggested that plant ferritin was induced in a range of 40–50-fold by iron overload through a transcriptional control. This has been confirmed by the report that iron treatment of plants was responsible for a rapid and transient accumulation of ferritin mRNA [11–13]; the ultimate demonstration that plant ferritin expression was transcriptionally regulated came from run-on experiments performed with isolated nuclei of soybean cells [11].

Despite this major difference between plants and animals in the regulation of iron-inducible expression of their ferritin genes, a weak effect of iron on the transcription of animal L-type ferritin genes has been noticed [21–23]. Also, post-transcriptional control of plant ferritin concentration in leaves, nodules and seeds during their development has been reported, and is likely to take place at the protein stability level [24–28]. Furthermore, the plant hormone abscisic acid (ABA) induces ferritin mRNA accumulation in leaves of iron-starved maize plants at a similar level to iron, but the ferritin protein abundance is much lower in response to ABA treatment than it is from iron overloading [12]. This raises the possibility that in plants, iron not only induces ferritin mRNA accumulation, but also controls the ferritin protein accumulation at the post-transcriptional level. In order to test this hypothesis, we have analyzed ferritin synthesis in maize leaves containing various concentrations of iron; modulation of leaf iron content has been made possible by the maize mutant *ys1* defective in the transport of Fe(III)-mugineic acid complexes at the plasma membrane of root cells [29,30]. Mugineic acid is a non-proteogenic amino acid able to chelate ferric iron with a high affinity constant. It is a graminaceous specific siderophore whose synthesis from *S*-adenosyl methionine, secretion in the rhizosphere and uptake by roots as a ferric chelate is induced by iron starvation [31–34]. Von Wirén et al. [35] have clearly demonstrated that in *ys1*, the release of phytosiderophores is similar to Fe-efficient maize cultivars, while the uptake of Fe-siderophores is much lower; a major consequence of the *ys1* mutation is to decrease the iron concentration in the leaves, and these authors concluded that this defect in the uptake system for Fe-phytosiderophores is responsible for the high susceptibility of *ys1* to Fe-deficiency-induced chlorosis. In this paper, we demonstrate that alteration in iron uptake by roots of the *ys1* genotype strongly decreases ferritin protein accumulation in the leaves but does not affect ferritin mRNA accumulation. This uncoupling between ferritin mRNA and protein levels reveals the existence of a post-transcriptional regulation of ferritin accumulation in plant cells in response to iron overloading.

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2. Materials and methods

2.1. Plant cultures

Maize seeds of a Fe-efficient variety (Alice plants) and a mutant, homozygote for the recessive *ys1* allele (*ys1* plants) were a gift of Dr. N. von Wirén [35]. Maize plantlets of both varieties were grown under hydroponic culture in a liquid iron-free medium as previously described [13]. After 7 days of iron starvation, plantlets were treated with iron according to two different procedures. Hydroponically grown plantlets (whole plantlets) were treated with a mixture of 500 μM iron-EDTA and a freshly prepared solution of 75 μM $FeSO_4$/150 μM trisodium citrate prepared according to Lobréaux et al. [13]. This Fe(III)-chelate mixture was added to the culture medium at the root level as previously described [13]. Alternatively, stems of de-rooted plants, cut at the level of the collar [36], were dipped into a freshly prepared solution of 500 μM $FeSO_4$/1 mM trisodium citrate prepared as described [13]. Whatever the iron treatment performed, leaf samples were harvested at different times after treatment, frozen in liquid nitrogen, and stored at −70°C.

2.2. Iron and protein concentration determination

Samples were mineralized and total iron concentration was estimated spectrophotometrically at 535 nm using bathophenanthroline, after reduction of the samples with thioglycolic acid as described [26]. Protein concentrations were determined according to Bradford [37].

2.3. Protein and RNA preparation and analysis

Preparation of total leaf protein, electrophoresis through a 15% SDS/polyacrylamide gel and immunodetection with polyclonal antibodies raised against maize seed ferritin or maize tubulin were performed as described [13]. Serum from the rabbit immunized with a 12 amino acid peptide located in the N-terminal region of the maize α-tubulin 1 was a generous gift of Dr. Joan Rigau (Dep. Genetica Molecular, CSIC, Barcelona, Spain).

RNA preparation and electrophoresis through a 0.8% agarose gel containing 6.6% formaldehyde were performed as described [13]. RNAs prepared from Alice and *ys1* samples were electrophoresed and transferred to nylon membrane (Hybond N^+, Amersham) simultaneously. Hybridization of Northern blots was carried out with a 620 bp *Sac*I-*Stu*I fragment from the ferritin cDNA FM1 [13] labelled with α-[^{32}P]dCTP using a random priming kit (Ready to Go, Pharmacia). Hybridizations were performed according to Fobis-Loisy et al. [15], overnight at 42°C in the presence of 50% formamide. After washing twice with 2×NaCl/Cit (300 mM NaCl, 30 mM sodium citrate), 0.1% SDS for 5 min at room temperature, and once with 0.1×NaCl/Cit, 0.1% SDS for 10 min at 42°C, the filters were autoradiographed at −70°C using Royal X-Omat films (Kodak).

3. Results

3.1. The ys1 maize mutant is deficient in leaf iron accumulation in comparison to the Fe-efficient genotype Alice

Plants homozygous for the recessive *ys1* allele, when grown in culture solution, poorly utilize chelated ferric iron in comparison with plants possessing at least one dominant allele for the *ys1* locus [29]. This *ys1* phenotype is due to a defect in the transport of Fe(III)-phytosiderophore complexes at the plasma membrane of root cells [29,30,35]. We have evaluated the impact of this root defect on the accumulation of iron in leaves of the *ys1* mutant when an excess of Fe(III) chelate was added to the culture medium, at the root level (Fig. 1a). *Ys1* leaf iron concentrations after 24, 48 and 72 h of treatment have been compared with iron concentrations in leaves of a Fe-efficient variety (Alice) treated under the same conditions (Fig. 1a). Iron content in leaves of plantlets increased up to 72 h after the beginning of iron supply by 2.5–3-fold whatever the genotype considered (Alice vs. *ys1*). However, iron concentration was 30–50% lower in *ys1* leaves than in Alice leaves. These results are consistent with those already reported in the literature [35].

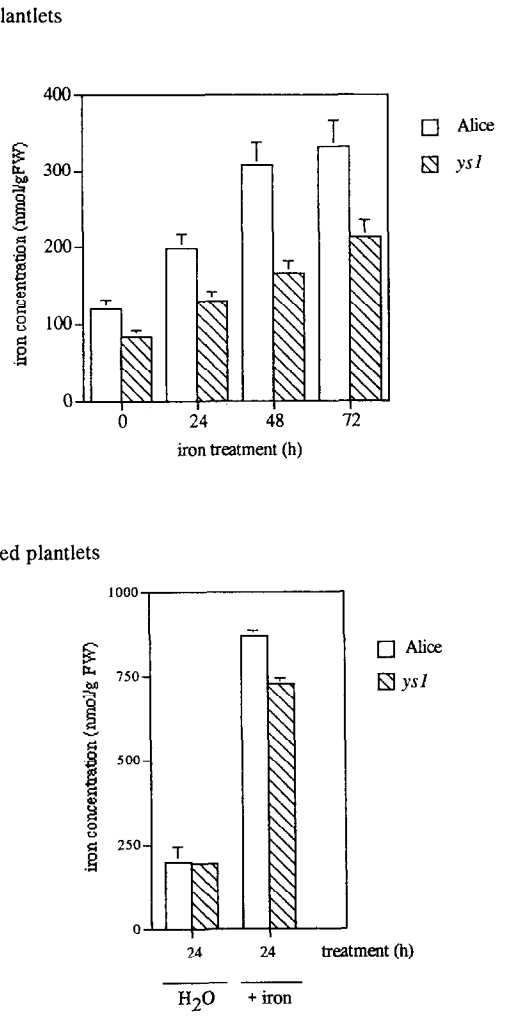

Fig. 1. Iron accumulation in leaves of iron-treated *ys1* plants compared to a Fe-efficient variety (Alice). a: Maize plantlets were incubated in a 500 μM Fe-EDTA/75 μM Fe-citrate solution. Iron concentrations were estimated in leaves prior to iron treatment (0) and 24, 48, and 72 h after iron supply to the culture medium as described in Section 2. Results are the mean of 3 experiments and bars indicate standard errors. b: Stems of de-rooted plantlets were dipped into a 500 μM iron-citrate solution for 24 h. As a control, de-rooted plantlets were incubated in water for 24 h instead of iron solution. Results are the mean of 3 experiments and bars indicate standard errors. Two independent experiments gave similar accumulation patterns.

Despite the large amount of iron on or in the roots of *ys1* plantlets, only a small amount reaches the xylem [30]. Therefore, the chlorotic *ys1* leaves can be regreened by dipping plantlets, whose root tips have been excised, in an iron-EDTA solution, indicating a physical way to bypass the root deficiency by loading iron directly in the xylem sap [29]. Consequently, we have tested if loading iron directly into the xylem sap could restore iron accumulation in *ys1* leaves. For this experiment, maize plantlets whose roots had been excised (de-rooted plantlets [36]) were dipped into a solution containing 500 μM iron-citrate, the main chemical form of iron found in the xylem sap of plants [38]. De-rooted *ys1* and Alice plantlets were dipped into an iron-citrate solution and leaf iron concentration was determined 24 h after iron supply (Fig. 1b). A significant increase in leaf iron concentration was observed. In this system, after 24 h of iron treatment,

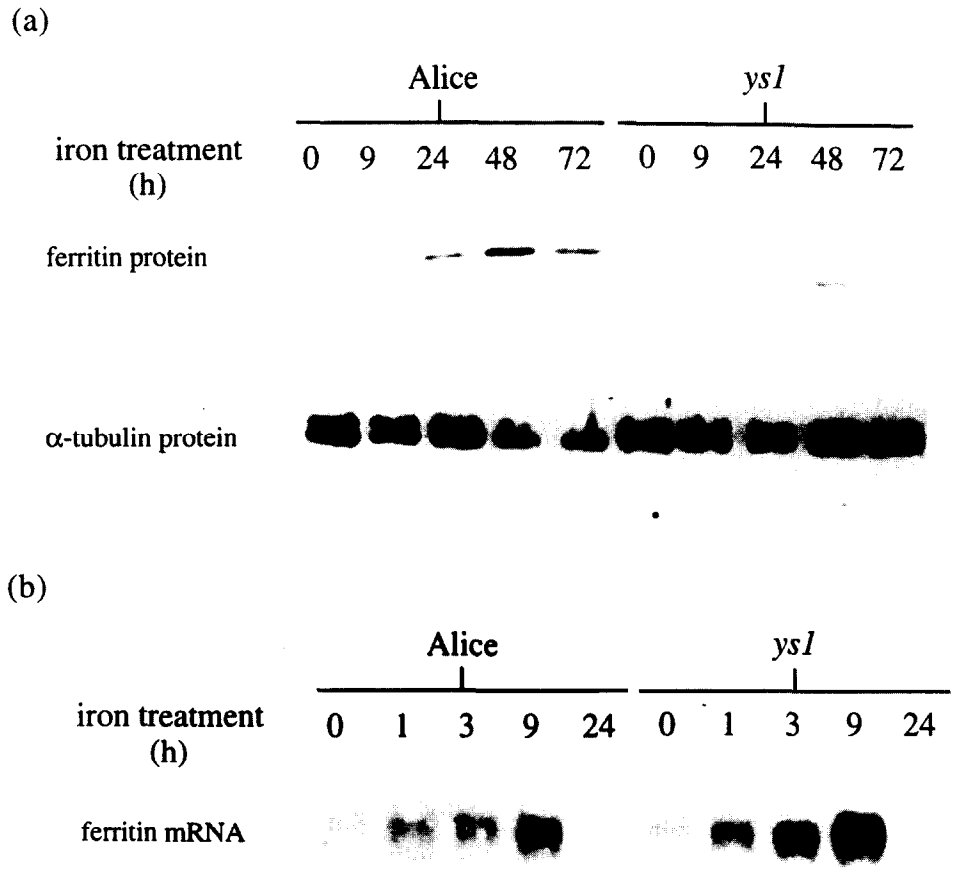

Fig. 2. Ferritin protein and mRNA abundance patterns in leaves of *ysl* and Alice plantlets reveal a post-transcriptional regulation of ferritin synthesis in response to iron. Time courses of ferritin and α-tubulin subunits, and ferritin mRNA abundance were determined for *ysl* and Alice. a: Immunodetection of ferritin and α-tubulin subunits electrotransferred to nitrocellulose membrane after separation of proteins through a polyacrylamide/SDS gel. 20 μg of total protein extracted from shoots prior to iron treatment (0) and 9, 24, 48, and 72 h after iron supply to the culture medium were loaded. Polyclonal antibodies raised against the maize ferritin or α-tubulin subunits were used at a dilution of 1/5000. Detection was achieved using the ICN Aurora Western blotting kit. b: 10 μg of total RNA extracted from shoots prior to iron supply (0) or various times after the iron treatment were electrophoresed through an agarose/formaldehyde gel and transferred to nylon membrane. Northern blots were analyzed with a maize ferritin cDNA ^{32}P-labelled probe [13]. Two independent experiments gave similar accumulation patterns.

the iron concentration is quite similar for both genotypes (about 750 nmol iron per gram of tissue fresh weight), and is consistent with previous observations using another maize genotype [36]. Iron concentration in de-rooted leaves is 2.5-fold higher than in whole Alice plantlets after 72 h of iron treatment (Fig. 1a). Indeed, cutting the roots allowed high iron concentrations to be obtained in leaf tissues whatever the genotype. This result indicates that root deficiency of *ysl* plantlets is responsible for reduced iron accumulation in leaf tissues and that it is possible to restore iron accumulation in *ysl* leaves to the same level as in a Fe-efficient genotype by cutting the roots. Despite this defect, however, we observed that this mutant is still able to accumulate iron in leaves when an excess of this element was supplied at the root level (Fig. 1a). This iron increase in *ysl* leaves can be explained by the existence of two other iron-uptake pathways independent of the siderophore pathway, namely the apoplastic pathway and the constitutive ferric-reductase/Fe^{2+} transporter pathway [39].

In conclusion, under our iron treatment conditions, *ysl* behaves as a Fe-deficient genotype compared to Alice. The use of the couple Alice/*ysl* therefore provides a system for the investigation of the effects of variations in total leaf iron concentration on ferritin protein accumulation.

3.2. *Post-transcriptional regulation of ferritin protein accumulation in response to iron overloading*

Several authors have reported that the concentration of animal and plant ferritin proteins increases proportionally with iron loading [40–43]. Thus, we have analyzed the ferritin protein level in leaves of whole *ysl* plantlets which, as described above after iron treatment, contained a reduced iron level in comparison with a Fe-efficient variety (Fig. 1a). The kinetics of ferritin protein accumulation in Alice leaves in response to an iron overload (Fig. 1b) was similar to that already reported with other iron efficient maize varieties (Fig. 2a [13]); the maximum level of protein accumulation was observed 48 h after the beginning of the iron treatment. In contrast, iron treatment of *ysl* plantlets under the same conditions led to a reduced level of ferritin protein accumulation (Fig. 2a). The maximum level of protein accumulation for *ysl* was also observed 48 h after the iron was supplied in the culture medium. Analysis of the same protein samples by Western blots using polyclonal antibodies raised against maize

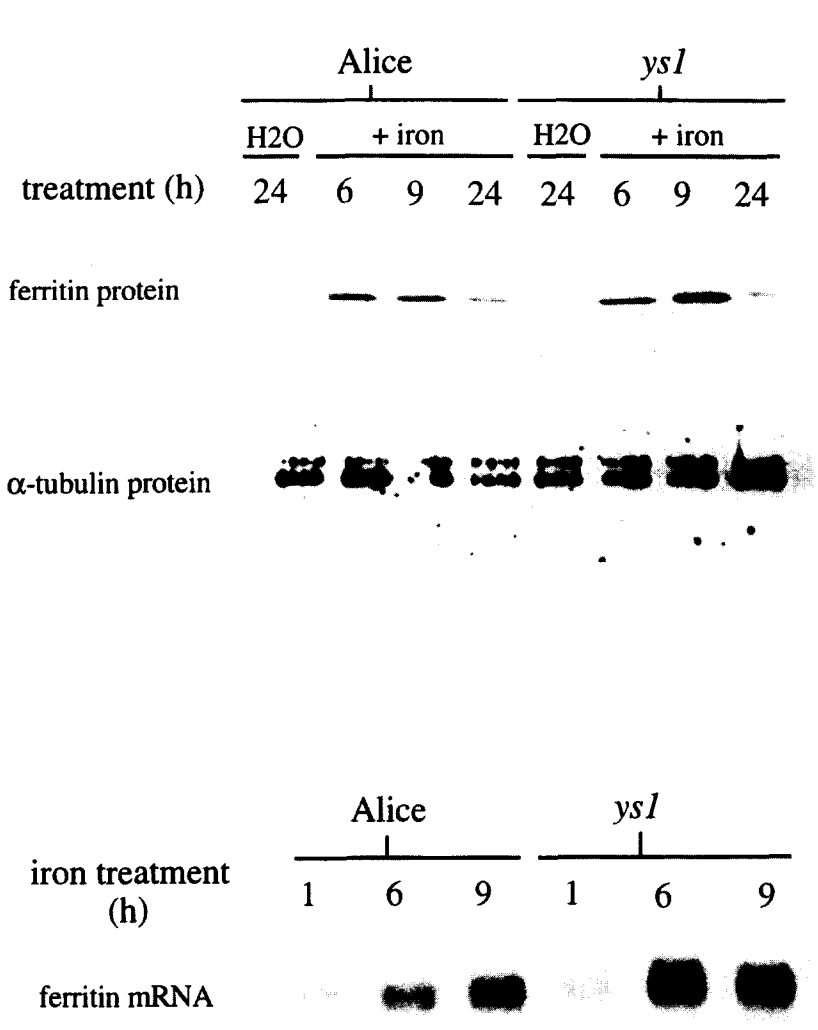

Fig. 3. Restoration of ferritin protein abundance in *ysl* de-rooted plantlets treated with excess iron. Time courses of ferritin and α-subunits and mRNA abundance were determined for *ysl* and Alice. Stems of de-rooted plantlets were dipped into a 500 μM iron-citrate solution for 1, 6, 9 and 24 h. As a control, de-rooted plantlets were incubated in water for 24 h instead of iron solution. Total protein and mRNA were extracted from shoots and analyzed as described in the legend of Fig. 2. Two independent experiments gave similar accumulation patterns.

α-tubulin revealed two polypeptides accumulated in similar amounts whatever the genotype considered (Alice vs. *ysl*), or the iron treatment duration (Fig. 2a). Despite the fact that two different polypeptides are recognized by the antibodies raised against α-tubulin for an unknown reason, it can be concluded that the lower level of ferritin protein accumulated in response to iron in the *ysl* mutant, compared to the Alice variety, is specific, and not just a general difference in the translational machinery between the two genotypes.

In cultured soybean cells, it has been demonstrated that the iron induction of ferritin synthesis was regulated at the transcriptional level [11]. In maize, ferritin mRNA accumulates in response to iron treatment [12,13,36]. It was therefore reasonable to predict that the differences in iron-induced ferritin protein accumulation observed between the varieties Alice and *ysl* (Fig. 2a) could result from differences in ferritin mRNA abundance. In order to test this hypothesis, the level of ferritin mRNA present in the two genotypes was determined by Northern blots (Fig. 2b). Surprisingly, kinetics and abundance of ferritin mRNA in iron-treated plantlets were found to be similar in both varieties, and to those already reported for other maize genotypes [12,13]. Ferritin mRNA accumulation was transient, peaking 9 h after the beginning of the iron treatment and returning to the basal level after 24 h (Fig. 2b). These data show that transduction pathways leading to ferritin mRNA accumulation in response to an iron overload were not disturbed in the *ysl* mutant. The uncoordinated changes in ferritin protein and mRNA abundances detected in *ysl* plantlets (Fig. 2) therefore reveals a post-transcriptional regulation of ferritin accumulation in response to iron loading which operates in addition to iron induction of ferritin mRNA accumulation.

We have determined that loading iron directly into the xylem sap of maize plantlets restored iron accumulation in the *ysl* genotype at a similar level as in a Fe-efficient variety (Fig. 1b). We therefore determined the effect of such a treatment on ferritin protein and mRNA abundances (Fig. 3). This de-rooted plantlet system has already been used to study the control of ferritin synthesis in response to iron [36]. In the present study, as observed for iron content, ferritin protein accumulation was restored in leaves of *ysl* de-rooted plantlets (Fig. 3a). Such a treatment led to a rapid and similarly tran-

sitory accumulation of ferritin protein in leaves of both genotypes after 6, 9 and 24 h of iron loading. This induction of ferritin accumulation was specific to the iron-citrate treatment, as determined by incubation of de-rooted plantlets in water instead of iron solution (Fig. 3a [36]). Probing the blots with α-tubulin antibodies as a control indicated that the effect of the various treatments on both genotypes was specific for ferritin accumulation in response to iron overload (Fig. 3a). Likewise, ferritin mRNA abundance and profile of accumulation in leaves of de-rooted plantlets were similar for both genotypes 9 h after iron supply (Fig. 3b). This result indicates that root deficiency of *ys1* plantlets is responsible for reduced ferritin protein accumulation in leaf tissues as it is possible to restore ferritin protein accumulation in *ys1* leaves to the same levels as in a Fe-efficient genotype by cutting the roots.

4. Discussion

Although contemporary plant and animal ferritins have arisen from a common ancestor and share highly conserved primary, secondary and tertiary structure [16,44,45], important differences have recently been pointed out concerning the mechanisms of regulation of their synthesis in response to an iron overload [11,12,36]. The major control of animal ferritin synthesis in response to iron is translational (see Section 1), while the main effect of iron in plants is to activate ferritin mRNA accumulation [11–13], which in at least one case has been demonstrated to be regulated at the transcriptional level [11]. This work is the first report of post-transcriptional control of plant ferritin accumulation in response to iron overloading. In order to demonstrate this, we have used the maize mutant *ys1*, which is defective in iron uptake due to impaired transport of iron-mugineic acid at the root level [35]. Our results showed that in response to treatment with an excess of iron, this mutant can be considered an Fe-deficient genotype which accumulates iron in leaves to lower levels than a Fe-efficient genotype (Alice).

In addition, as in Fe-efficient genotypes, ferritin synthesis in the *ys1* mutant is controlled at the mRNA level (Fig. 2b), demonstrating that the *ys1* mutation does not affect transduction pathways responsible for ferritin mRNA accumulation in response to iron overloading. We have, however, observed an uncoordinated ferritin protein and mRNA accumulation in *ys1* leaves after iron supply at the root level (Fig. 2). In contrast, loading iron-citrate directly in the xylem sap of the *ys1* mutant restored ferritin protein accumulation and correlation with ferritin mRNA abundance (Fig. 3). However, no experimental evidence indicating whether it is possible to uncouple ferritin mRNA and ferritin protein accumulation in the maize wild type Alice is presently available. It is therefore unknown whether the regulation of iron-induced ferritin synthesis which occurs both at the mRNA and at the protein level is specific of the *ys1* mutant or not. Where this post-transcriptional control occurs in *ys1*, translation and/or protein turn-over, in response to iron as demonstrated here remains to be determined.

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