the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2009) **57**, 400–412 doi: 10.1111/j.1365-313X.2008.03698.x

Ferritins control interaction between iron homeostasis and oxidative stress in Arabidopsis

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Received 24 July 2008; revised 27 August 2008; accepted 4 September 2008; published online 28 October 2008.
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Summary

Ferritin protein nanocages are the main iron store in mammals. They have been predicted to fulfil the same function in plants but direct evidence was lacking. To address this, a loss-of-function approach was developed in Arabidopsis. We present evidence that ferritins do not constitute the major iron pool either in seeds for seedling development or in leaves for proper functioning of the photosynthetic apparatus. Loss of ferritins in vegetative and reproductive organs resulted in sensitivity to excess iron, as shown by reduced growth and strong defects in flower development. Furthermore, the absence of ferritin led to a strong deregulation of expression of several metal transporters genes in the stalk, over-accumulation of iron in reproductive organs, and a decrease in fertility. Finally, we show that, in the absence of ferritin, plants have higher levels of reactive oxygen species, and increased activity of enzymes involved in their detoxification. Seed germination also showed higher sensitivity to pro-oxidant treatments. Arabidopsis ferritins are therefore essential to protect cells against oxidative damage.

Keywords: ferritin, iron homeostasis, oxidative stress, iron storage, iron transporter, Arabidopsis.

Introduction

Iron is essential for all living cells because it is a constituent of a number of important macromolecules, including those involved in respiration, photosynthesis, DNA synthesis and metabolism. However, excess free iron is potentially detrimental to the cell because of its propensity to react with oxygen, generating harmful free radicals by the Fenton reaction. Because of this dual nature, maintenance of correct labile iron levels is a critical component in preserving homeostasis. Thus, elaborate systems tightly control the concentration of available iron. Iron homeostasis throughout the life cycle is a dynamic process resulting from integrated regulation of expression of the various genes encoding proteins acting in transport, utilization and storage of this metal.

Ferritins are a class of universal 24-mer multi-meric proteins, present in all kingdoms of life and able to accommodate several thousand iron atoms in their central cavity (Harrison and Arosio, 1996). In animals, the primary function of ferritins inside cells is to store iron, and to deliver the metal for metabolism when needed. During normal growth and development, some specialized cells accumulate iron and ferritin for use by other cells later in development, exemplified by the erythrocytes of embryos and the liver of the fetus (Theil, 2003). Furthermore, a mutant lacking ferritin has been generated in mice, and homozygous animals die *in utero*, whereas heterozygotes exhibit signs of mild iron deficiency (Thompson *et al.*, 2003). When stored in ferritins, iron cannot react with oxygen. Thus, a secondary function for ferritin may be protection against free iron-induced production of reactive oxygen species (ROS). Indeed, in bacteria, deletion of ferritin genes increases oxidant sensitivity (reviewed by Theil *et al.*, 2006).

In plants, most of the hypotheses formulated regarding ferritin functions are based on correlations between localization of the proteins and their expression in response to environmental factors and developmental stages (Briat and Lobréaux, 1997; Briat *et al.*, 1999). In seeds, ferritins are widely proposed to be the major iron-storage form, and to release and provide iron to iron-containing proteins after germination. Seed ferritins are therefore essential for correct

building of the photosynthetic apparatus, and as such are involved in the autotrophy/heterotrophy transition (Lobréaux and Briat, 1991). In leaves, it was hypothesized that ferritin is an iron source at early stages of development for the synthesis of iron-containing proteins involved in photosynthesis (Briat and Lobréaux, 1997; Lobréaux and Briat, 1991; Theil and Hase, 1993).

To provide direct evidence regarding ferritin functions in plants, we developed a loss-of-function approach in Arabidopsis. Arabidopsis ferritins are encoded by four genes named *AtFer1–4. AtFer2* is the only gene that is expressed in seeds, whereas *AtFer1*, *AtFer3* and *AtFer4* are expressed in vegetative and reproductive tissues (Petit *et al.*, 2001). In this study, we isolated and characterized knockout mutants lacking either the seed isoform FER2, or the three isoforms of vegetative tissues, FER1, 3 and 4. Our results shed new light on the functions of plant ferritins. They are not an iron source for development, but are of key importance in the defence machinery against free iron-induced oxidative stress.

Results

Isolation of ferritin-lacking Arabidopsis mutants

A T-DNA insertion line in *AtFer2* (named *fer2*) was isolated (Appendix S1) and analyzed. Ferritin content was monitored in Col and *fer2* seeds by Western blot analysis (Figure 1a). No signal was detected in *fer2*, indicating firstly that *AtFer2* expression is completely abolished in this mutant, and secondly that FER2 protein is the only ferritin subunit expressed in seeds. In order to elucidate ferritin function in vegetative organs, knockout mutants in the three other ferritin genes were used. A T-DNA insertion line for the *AtFer1* gene has been described previously (Dellagi *et al.*, 2005; Murgia *et al.*, 2007). T-DNA insertion lines in the *AtFer3* and *AtFer4* genes were isolated (Appendix S1). These mutants were used to create a triple *fer1 fer3 fer4* mutant, abbreviated as *fer1-3-4* below (Figure S1).

Ferritin accumulation was investigated by Western blot using leaves from 3-week old Col, *fer1*, *fer2* and *fer1-3-4* plants grown on soil and irrigated with water or 2 mM Fe-ethylenediaminedi(o-hydroxyphenylacetic) acid (EDDHA) (Figure 1b). In Col and *fer2*, two polypeptides of approximately 28 and 26.5 kDa were detected. These proteins are over-accumulated in plants treated with iron, suggesting that they correspond to ferritin proteins. The mature FER1 subunit migrated at 28 kDa when expressed in *Escherichia coli* (data not shown), indicating that the immunodetected 28 kDa polypeptide corresponds to the mature ferritin subunit. The 26.5 kDa peptide probably corresponds to a processed form of ferritin. Such post-translational processing of plant ferritin subunits has been reported in other species (Laulhère *et al.*, 1989; Lobréaux and Briat, 1991;

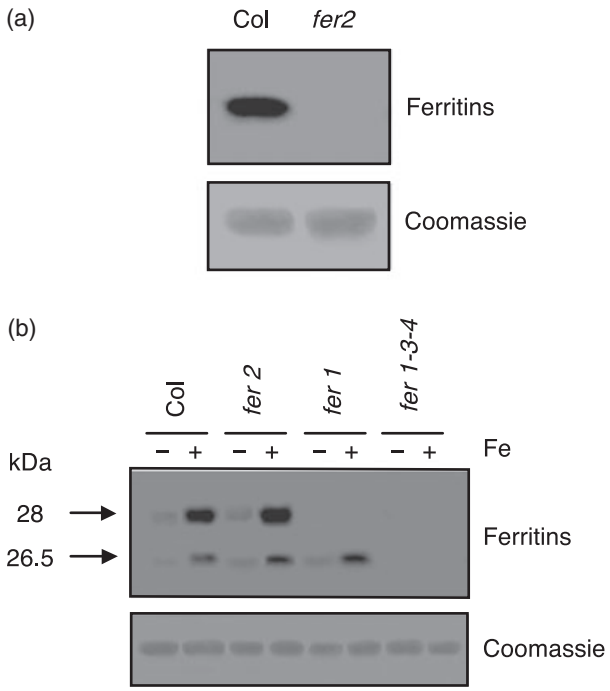


Figure 1. Isolation of ferritin-lacking Arabidopsis mutants.
(a) Molecular characterization of the *fer2* mutant. Ferritin accumulation in Col and *fer2* seeds was analysed by Western blotting. Total proteins (10 μg) were subjected to SDS–PAGE and transferred onto a PVDF membrane. Immunodetection was performed using anti-FER1 serum. Coomassie brilliant blue staining is shown as a loading control.
(b) Molecular characterization of the *fer1-3-4* mutant. Wild-type (Col) and mutants *fer2*, *fer1* and *fer1-3-4* were grown on soil and irrigated with (+) or without (–) 2 mM Fe-EDDHA. Total protein extracts (10 μg) from leaves were loaded on each lane. Western blotting was performed as described in (a). Arrows indicate the position of the unprocessed (28 kDa) and processed (26.5 kDa) isoforms. A gel stained using Coomassie brilliant blue is shown as a loading control.

Masuda *et al.*, 2001). In *fer1*, only the 26.5 kDa polypeptide was detected, indicating that FER1 exists only as a 28 kDa protein. The relative abundance of the 26.5 kDa polypeptide was similar in Col, *fer1* and *fer2*, suggesting that FER3 and FER4 subunits accumulate in leaves as 26.5 kDa processed subunits. Ferritin accumulation was also investigated in flowers of plants irrigated with water or 2 mM Fe-EDDHA. The abundance of ferritin subunit in flowers was similar to those determined in leaves (data not shown). In neither organ was signal detected for *fer1-3-4*, indicating that the triple mutant is devoid of ferritins in the shoot.

Seed ferritins are not a major iron store, but play a protective role against oxidative stress during germination

The *fer2* mutant was used to determine the role of ferritin in early steps of seedling development. During germination, FER2 abundance rapidly decreased in wild-type seeds (Figure 2a). No ferritin was detected in *fer2* seeds after 12 or 24 h of germination, indicating that there was no change in

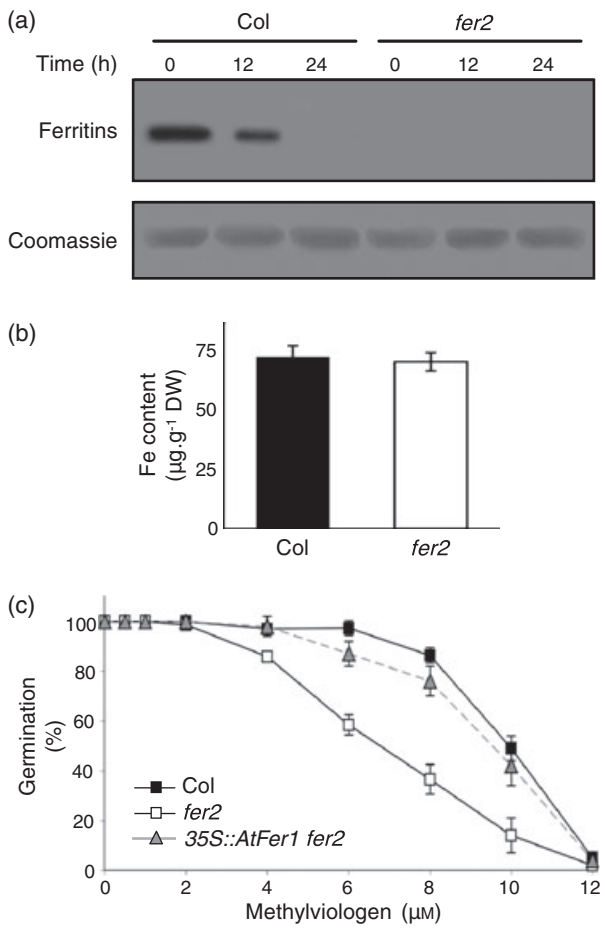


Figure 2. Seed ferritin protects against oxidative stress during germination, but is not the major iron store.
(a) Ferritin degradation after germination. Col and *fer2* seeds were sown on de-ionized water–agar medium, stratified for 2 days in the dark, then transferred to a growth chamber. Seeds were harvested before (0), 12 and 24 h after illumination. Ferritin immunodetection was performed as described in Figure 1(a).
(b) Iron content in dry seeds of Col and the *fer2* mutant. Means and standard deviations were calculated from measurements performed on six co-harvested seed pools.
(c) Dose effect of MV on seed germination. Col, *fer2* and *35S::AtFer1 fer2* seeds were sown as described in (a). The germination rate was scored after 72 h. Each data point corresponds to the mean of five replicates of 200 seeds ± SD.

expression of the other FER subunits. The germinating capacity of the seeds was assayed on media containing various amounts of iron (0, 50 and 300 µM Fe(III)-EDTA). No difference in germination was observed between Col and *fer2* seeds, regardless of the concentration of iron (Figure S2), and development of the seedling was similar for both genotypes. Interestingly, the seed iron content in Col and *fer2* was identical (Figure 2b). The proportion of iron stored in ferritin in Arabidopsis seeds was then estimated in Col. The amount of ferritin was found to be around 1 ng μg^{-1} total protein, using purified recombinant FER2 protein as a standard. Assuming that a 24-mer native ferritin may store on average 2000 Fe atoms (Laulhère *et al.*, 1988), we estimated that Arabidopsis ferritins store only about 5% of the total seed iron. Together, these results indicate that ferritins do not constitute the major seed iron pool, and that the absence of ferritins does not have an impact on germination.

By storing iron in a safe form, ferritins have been proposed to be involved in the protection against oxidative stress. To determine whether this occurred in seeds, Col and *fer2* germination was investigated under pro-oxidant conditions, by sowing on a medium containing methylviologen (MV). Increasing MV concentration led to a decrease in the germination rate in both Col and *fer2*, but *fer2* seeds displayed higher sensitivity to MV (Figure 2c). A line overexpressing *AtFer1* cDNA under the control of the 35S promoter was crossed with *fer2*. The rate of germination of *35S::AtFer1 fer2* seeds was close to that of Col, showing that the higher MV sensitivity was due to the absence of ferritin in seeds.

Our results demonstrate that ferritins protect the seed from free-iron-mediated oxidative stress, but do not constitute the major iron pool available for proper development of the young plantlet.

Absence of leaf ferritins leads to a decrease in leaf growth and CO_2 fixation

The impact of the absence of ferritin on vegetative growth was analyzed. Col and *fer1-3-4* genotypes were grown on soil and irrigated with water or 2 mM Fe-EDDHA. The amount of dry matter was measured from germination to bolting (Figure 3a). On water, the growth of the two genotypes was equivalent during the whole period. When irrigated with Fe-EDDHA, Col and *fer1-3-4* exhibited the same growth for a period of about 30 days after sowing. Thereafter the amount of dry matter in iron-treated Col plants was significantly greater than that in plants irrigated with water. By contrast, *fer1-3-4* growth was drastically reduced by the iron treatment. Before bolting, about 50 days after sowing, the amount of dry matter in the mutant was 65% lower than that of the wild-type. However, the developmental stage of *fer1-3-4* appeared similar to that of Col. The number of leaves was the same but their size was clearly reduced (Figure 3b). The iron content in leaves was determined. Iron irrigation led to increased iron content in the leaves of both genotypes to a similar extent (data not shown).

To ensure that the mutant growth defect was due to the absence of ferritin in leaves, *fer1-3-4* was transformed with the full-length *AtFer1* cDNA under the control of the 35S promoter. Three independent lines were selected, and the amount of dry matter in iron-irrigated transformed *fer1-3-4* lines was determined. The three *35S::AtFer1*-transformed *fer1-3-4* lines exhibited similar growth to Col (Figure 3a for *35S::AtFer1 fer1-3-4* line 4). It can therefore be concluded that the absence of ferritin leads to a strong decrease in

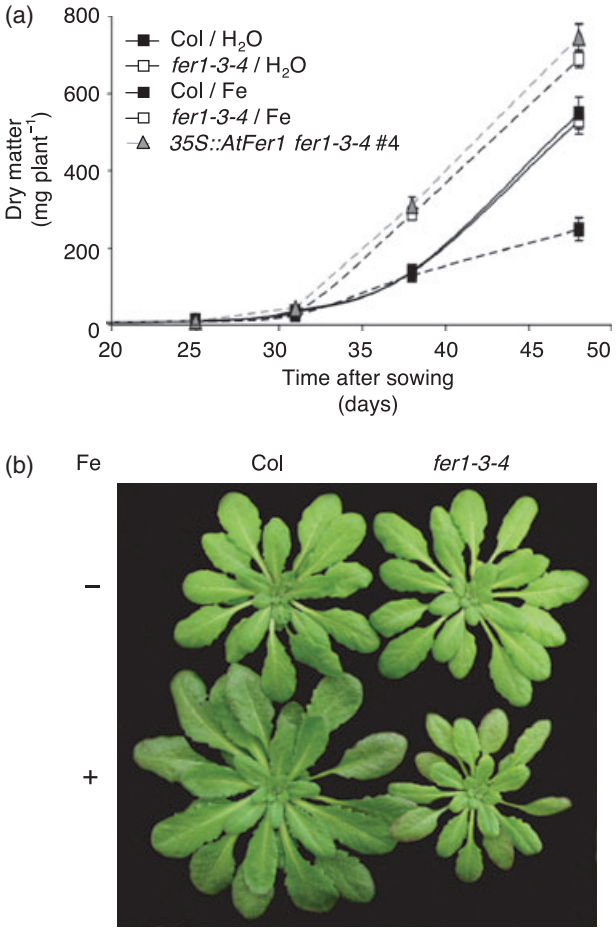


Figure 3. Disruption of leaf-expressed ferritin genes alters rosette development in excess iron.
(a) Growth of Col and *fer1-3-4*. Plants were grown on soil and irrigated with water or 2 mM Fe-EDDHA. Rosettes were collected at various time points after sowing until bolting (day 48), and dry matter was measured. *fer1-3-4* transformed with a *35S::AtFer1* construct was used to demonstrate complementation. Values are means ± SD (n = 6).
(b) Rosette phenotype of *fer1-3-4*. Plants were grown on soil and irrigated (+) or not (–) with 2 mM Fe-EDDHA. The plants shown are 45 days old.

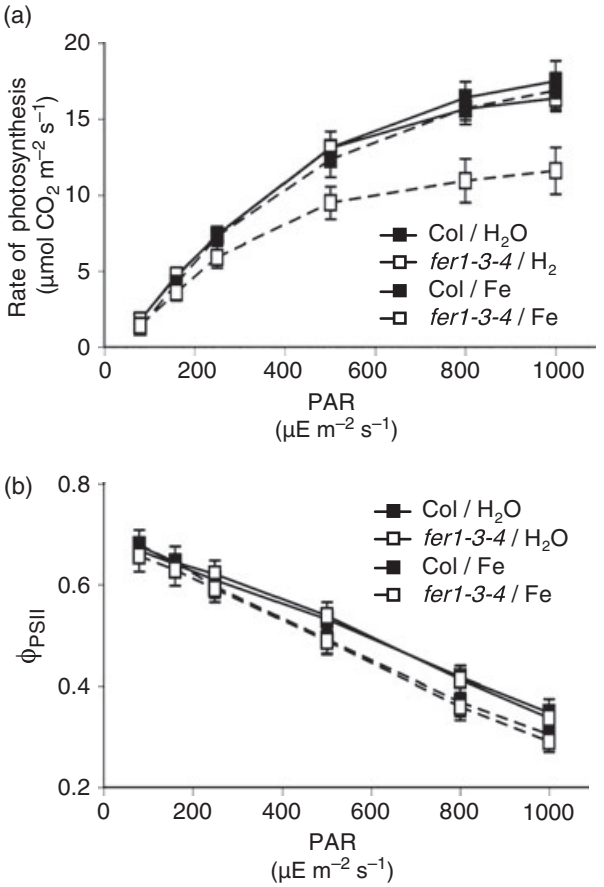


Figure 4. Photosynthesis in the *fer1-3-4* mutant.
(a) Response of the net photosynthesis rate to incident photosynthetic active radiation of Col and *fer1-3-4* leaves. Plants were grown on soil and irrigated with water or 2 mM Fe-EDDHA. Values are means ± SD (n = 10).
(b) Quantum yield of PSII (ϕ_{PSII}) as a function of incident photosynthetic active radiation. Plants were grown as described in (a). Values are means ± SD (n = 10).

growth of the rosette when plants are irrigated with iron, a favourable condition for Col as evidenced by its increased biomass.

In order to obtain further insights into the mechanisms underlying the strong growth reduction of *fer1-3-4* when irrigated with iron, the photosynthetic efficiency in the triple mutant and Col was compared. Net photosynthesis was determined by measuring CO_2 fixation in leaves of 5-week-old plants irrigated with water or 2 mM Fe-EDDHA. Measurements were performed at light intensities ranging from 80 to 1000 μE m^{-2} sec^{-1} (Figure 4a). When irrigated with water, both genotypes exhibited the same CO_2 fixation capacity. Under iron irrigation, the CO_2 fixation capacity of *fer1-3-4* decreased by more than 25% relative to the wild-type at 1000 μE m^{-2} sec^{-1}.

Both genotypes exhibit the same stomatal conductance (data not shown). The strong CO_2 fixation decrease was not due to differences in total chlorophyll or alterations of photosynthetic complexes (Figure S3). The chlorophyll/protein ratio was similar in both genotypes, and was unchanged in response to iron irrigation. Similarly, the relative abundances of photosystem I and II polypeptides, cytochrome b_6/f, ferredoxin and NFU2, a plastidial protein involved in iron–sulfur assembly, were unaffected by the mutation in ferritin genes. Chlorophyll fluorescence measurements were used to determine the efficiency of electron flux through the electron transport chain (Maxwell and Johnson, 2000; Oxborough, 2004). The electron flux through PSII (ϕ_{PSII}; Genty *et al.*, 1989) was not affected by iron treatment in either genotype, and was equivalent for Col and *fer1-3-4* (Figure 4b).

In conclusion, when grown on iron, the photosynthetic electron transfer chain was not altered (based on chlorophyll

and protein abundance and ϕ_{PSII} measurements), but was used less efficiently by the mutant for CO_2 assimilation, and consequently led to a decrease in biomass production.

Absence of ferritin impairs flower development

The development of Col and *fer1-3-4* plants proceeded similarly, and bolting occurred at the same time. However, when the mutant irrigated with iron reached the reproductive stage, a dramatic impact on flower development was observed, while wild-type flowers developed normally (Figure 5a). The developmental defects of *fer1-3-4* were most visible in the oldest inflorescences, where flowers exhibited numerous alterations. Silique development was arrested in most of the mutant flowers (Figure 5a). The reproductive organs appeared fairly normal but stunted. The stamen filaments rarely elongated to position the anthers above the stigma, and stigmatic papillae did not mature as seen in wild-type flowers. The anthers shed very few but normal-looking pollen grains (data not shown). These strong defects in flower development led to sterility in the most severe cases. The impact of the absence of ferritins on fruit development was quantified by determining the seed yield of Col and *fer1-3-4* grown under various iron-nutrition conditions (0, 0.5, 1 or 2 mM Fe-EDDHA). Increasing the iron concentration led to an increase in seed production for Col, but strongly reduced the seed yield for *fer1-3-4* (Figure 5b) by between 15% (on 0.5 mM Fe-EDDHA) and 70% (on 2 mM Fe-EDDHA). To confirm that the strong seed yield reduction was related to the lack of ferritin, *fer1-3-4* was transformed with the full-length *AtFer1* cDNA under the control of the 35S promoter. Three independent lines were analyzed (Figure 5b). The overexpression of FER1 in *fer1-3-4* increased the seed production threefold when compared to *fer1-3-4*. This shows that the alterations in flowers and the reduction of seed yield are consequences of the absence of ferritins.

Given the strong growth reduction of the *fer1-3-4* rosette when irrigated with iron (Figure 3), alteration of flower development could be a consequence of global deregulation of iron homeostasis in the leaves. To test this hypothesis, reciprocal grafting experiments between Col and *fer1-3-4* were performed (Figure 6). When a floral stalk from Col was grafted onto a mutant rootstock, the flowers were of the wild-type phenotype. However, when a *fer1-3-4* floral stalk was grafted onto a wild-type rootstock, the fruits exhibited strong alterations. This result indicates that the flower phenotype was not due to growth defects of the leaves, but was more likely related to the absence of ferritins in the floral stalk.

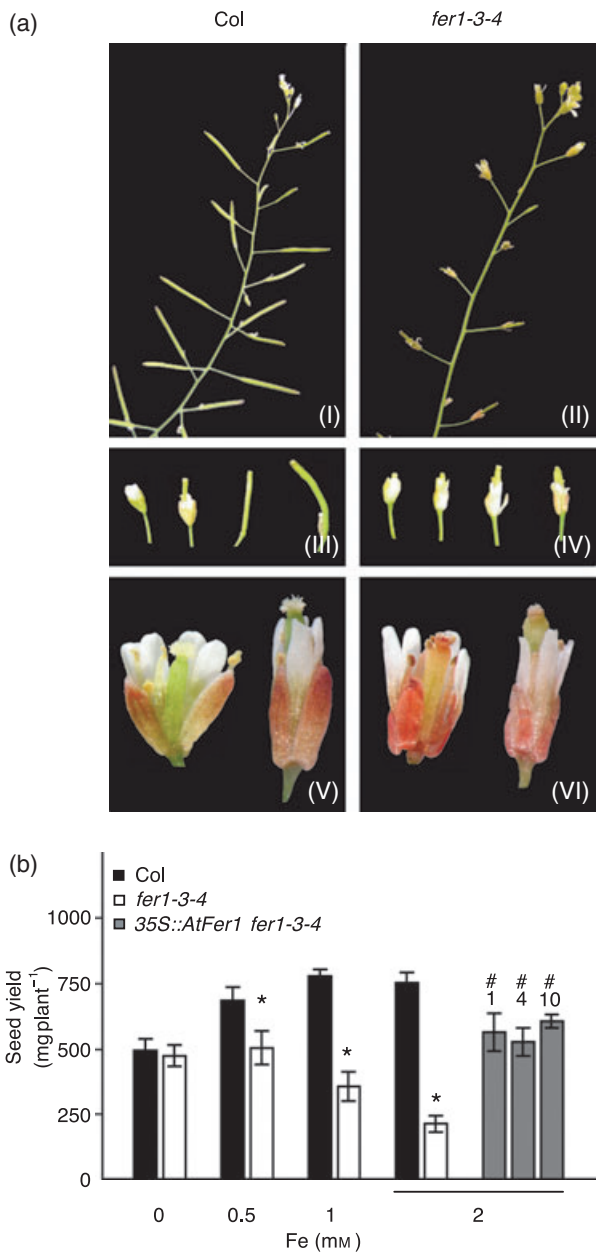


Figure 5. Pleiotropic alterations during the fruit formation in the *fer1-3-4* mutant irrigated with iron.
(a) Phenotype of *fer1-3-4* mutant reproductive organs. Plants were grown on soil and irrigated with 2 mM Fe-EDDHA. (I,II) Floral stalks, (III,IV) early steps of fruit development, (V,VI) unfertilized and fertilized flowers.
(b) Seed production is decreased in *fer1-3-4* irrigated with iron. Plants were grown on soil and irrigated with water (0) or 0.5, 1 or 2 mM Fe-EDDHA during the entire plant lifetime. Three independent lines of *fer1-3-4* transformed with a *35S::AtFer1* construct (lines 1, 4 and 10) were used to demonstrate complementation. Seeds were collected on individual plants and weighed. Values are means ± SD (n = 24). Significant differences (P < 0.005) between Col and *fer1-3-4* are indicated by an asterisk.

Disruption of ferritin genes alters iron transport in the floral stalk

The grafting experiments indicated that the flower defects in *fer1-3-4* originated from alterations in the floral stalk. The total iron content in the stem, unfertilized and fertilized flowers from Col and *fer1-3-4* irrigated with water or 2 mM Fe-EDDHA was determined (Figure 7a). When irrigated with

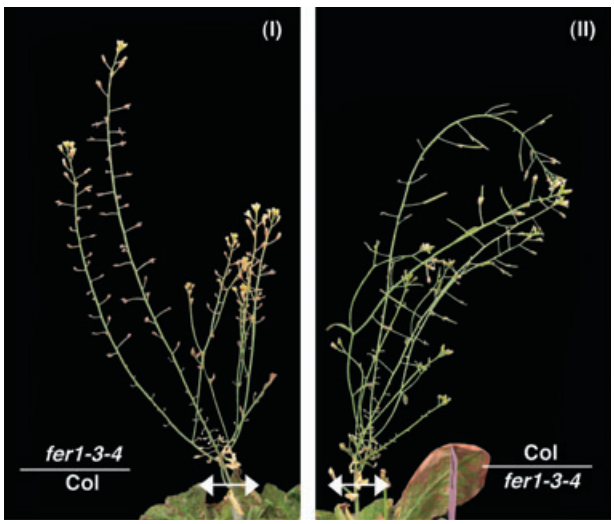
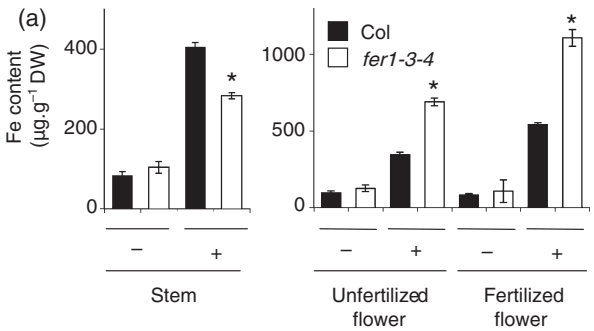


Figure 6. The flower phenotype of *fer1-3-4* is independent of leaves.
Reciprocal grafting experiments were performed using bolted Col and *fer1-3-4* plants grown on soil and irrigated with 2 mM Fe-EDDHA. In (I), a Col rosette was used as the rootstock and an *fer1-3-4* floral stalk as the scion. In (II), an *fer1-3-4* rosette was used as the rootstock and a Col floral stalk as the scion. Arrows indicate the grafting junctions.



(b)

Gene	AGI	Stem		Flowers	
		Col	*fer1–3–4*	Col	*fer1–3–4*
NRamp1	At1g80830	–3.7	–1.5	–1.6	2.2
NRamp2	At1g47240	–2.1	–2.5	–1.1	1.0
NRamp3	At2g23150	20	2.0	4.4	–1.2
NRamp4	At5g67330	1.5	–1.5	2.2	2.1
NRamp5	At4g18790	43	30	9.6	2.7
NRamp6	At1g15960	2.0	6.0	37	6.2
YSL1	At4g24120	2.4	1.3	1.3	–2.3
YSL2	At5g24380	–2.2	–4.9	–130	–281
YSL3	At5g53550	1.6	8.9	3.9	4.0
YSL4	At5g41000	7.5	1.8	2.7	–6.6
YSL5	At3g17650	–1.3	2.1	1.1	4.0
YSL6	At3g27020	1.2	–1.2	1.5	1.2
YSL7	At1g65730	8.1	1.1	2.7	–6.0
YSL8	At1g48370	–1.3	–2.2	–1.7	–2.9
IRT1	At4g19690	1.4	–40	2.1	–468
IRT2	At4g19680	1.2	–104	1.5	–9.4
ZIP1	At3g12750	–1.4	–2.0	1.9	1.3
ZIP4	At1g10970	1.3	3.1	1.2	1.8
ZIP5	At1g05300	13	3.6	–2.5	–1.4
ZIP6	At2g30080	–1.4	–1.6	–2.2	–1.9
ZIP9	At4g33020	7.6	6.9	2.0	1.3
ZIP12	At5g62160	7.2	18	1.7	–2.1
Pic1	At2g15290	1.6	1.8	6.7	14
Vit1	At2g01770	–1.5	1.4	1.1	1.0

Induction ≥ 20 | 20 > Repression ≥ 2
20 > Induction ≥ 2 | Repression ≥ 20

Figure 7. Ferritin disruption leads to alteration of iron allocation in the floral stalk.
(a) Iron Content Stems, unfertilized and fertilized flowers were harvested from Col and *fer1-3-4* grown on soil and irrigated with water (–) or 2 mM Fe-EDDHA (+).Values are means ± SD (n = 10). Significant differences between the two genotypes ($P < 0.005$) are indicated by asterisks.
(b) Ferritin disruption leads to deregulation of iron transport-related genes. Stems and flowers were collected from Col and *fer1-3-4* mutant plants irrigated with water or 2 mM Fe-EDDHA. Relative transcript levels were determined for each transporter gene by quantitative RT-PCR using the *SAND* gene as a control. The ratios between relative transcript level values for samples from plants irrigated with iron and samples from plants irrigated with water were calculated. These ratios show the fold change in expression of each gene between iron-irrigated and water-irrigated plants. The data shown correspond to the mean of three biological replicates (Appendix S2).

water, there was no significant difference in iron content between the genotypes in the organs analyzed. On 2 mM Fe-EDDHA, the total iron content of the stem was significantly lower in the mutant. By contrast, the iron content in unfertilized and fertilized flowers was higher in *fer1-3-4* compared to Col. Similar results were obtained with other iron chelates (i.e. 2 mM Fe-EDTA and 2 mM Fe-citrate; data not shown). The pattern of ferritin expression and iron localization in flowers was then investigated (Figure S4 and Appendix S1). The localization of *AtFer1*, *AtFer3* and *AtFer4* expression was investigated using transgenic plants carrying the glucuronidase gene (GUS) under the control of the promoter region of each gene. The pattern of iron accumulation was visualized using Perl staining. Our results indicate that, in the mutant flower, iron is over-accumulated in organs in which ferritin expression is strong in the wild-type (sepals, stamens and style of the stigma).

The difference in iron allocation between stem and flowers in the mutant when grown on iron (Figure 7a) prompted us to study the expression of genes related to iron transport, in order to identify the molecular mechanisms underlying this alteration. Quantitative RT-PCR analysis was performed on RNA extracted from stems and flowers of Col and *fer1-3-4* irrigated with water or 2 mM Fe-EDDHA (Table S2). The genes analyzed were selected from families known or predicted to be involved in iron transport, namely members of the four families *IRT*, *ZIP*, *NRamp* and *YSL*, and additional two single genes *Vit1* and *Pic1* (Briat *et al.*, 2007; Colangelo and Guerinot, 2006; Curie and Briat, 2003; Duy *et al.*, 2007; Guerinot, 2000; Kim and Guerinot, 2007; Kim *et al.*, 2006). When plants were irrigated with water, no significant difference in expression of the transporter genes was observed between the two genotypes regardless of the organ analyzed (Appendix S2). For each gene, the induction (or repression) factor in response to iron was calculated as

the ratio of expression between iron- and water-irrigated plants in stems and flowers for both genotypes (Figure 7b).

Interestingly, iron treatment led to changes in expression for most of the genes studied in the *fer1-3-4* mutant when compared to Col. Among these genes, *NRamp3*, *YSL4* and *YSL7* (in both organs), *ZIP5* (in stems) and *NRamp5* (in flowers) were induced in response to iron in Col, and induction was lower in *fer1-3-4*. *NRamp6* and *ZIP4* (in the stem) and *Pic1* (in flowers) were induced in response to iron, and upregulated in *fer1-3-4*. *NRamp1* (in flowers) was repressed in response to iron in Col, but induced in *fer1-3-4*. *YSL5* was not regulated by iron treatment in Col in either flowers or the stem, but was upregulated in response to iron in *fer1-3-4*. In flowers of both genotypes YSL3 was induced in response to iron but in the stem this gene was upregulated only in *fer1-3-4*. In the stem of both genotypes ZIP12 was induced in response to iron but was downregulated in the flowers of *fer1-3-4*. Finally, three additional genes were considered. While expression of NRamp4 was slightly unregulated by iron in both organs of the wild-type, it was downregulated in *fer1-3-4* stem. Expression of IRT1 and IRT2 was low in the wild-type and highly downregulated in *fer1-3-4*. These data indicate that the expression of genes related to iron transport is strongly affected in the absence of ferritin.

Alteration of iron homeostasis in fer 1-3-4 activates ROS-scavenging mechanisms

The absence of ferritin could lead to an increase in free iron, a potential pro-oxidant, through the Fenton reaction. To investigate a putative relationship between the absence of ferritin and the occurrence of oxidative stress, the activities of several enzymes known to be involved in ROS detoxification were measured in leaves and flowers from Col and *fer1-3-4* irrigated with water or iron. Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) were selected for these measurements. In both genotypes, iron excess led to an increase in APX, SOD and CAT activities (data not shown).

Interestingly, when irrigated with water, CAT, GR and APX activities were significantly higher in leaves and flowers of *fer1-3-4* when compared to Col (Figure 8a). These results indicate that, although the total iron content was equivalent in Col and *fer1-3-4* when irrigated with water (Figure 7a for flowers, data not shown for leaves), the absence of ferritin enhanced oxidative stress responses, probably because of an increase in free-iron-induced ROS production. To investigate this hypothesis, ROS accumulation and localization was analyzed in flowers using the fluorescent compound carboxy-dichloro-fluorescein (DCFDA). The fluorescence was more than twice as high in the flowers of *fer1-3-4* compared to those of wild-type (Figure 8b). The same results were obtained in leaves using diaminobenzidine as another ROS production marker (data not shown).

To estimate the redox state of leaves and flowers, the two anti-oxidant molecules glutathione and ascorbate were quantified, and their redox state was determined (Figure 8c). When irrigated with water, the concentrations of total ascorbate and glutathione were similar in leaves and flowers of both genotypes, as were the proportions of these compounds present in oxidized forms. Finally, the deleterious effects of ROS production on cell integrity were estimated by measuring malondialdehyde production, a marker of lipid peroxidation. The production of malondialdehyde was similar in Col and *fer1-3-4* (Figure 8d).

These data clearly shows that, under water irrigation, the absence of ferritin leads to ROS production, probably through the Fenton reaction. *fer1-3-4* upregulated ROS-scavenging mechanisms: enzymatic ROS detoxification activities were enhanced, reduced ascorbate and glutathione pools were maintained at levels similar to those of Col, and ROS-induced damage was avoided, as determined by assessment of lipid peroxidation.

Discussion

Despite a wealth of information on ferritin biochemistry and expression in plants, the main function of this iron-storage protein in the physiology and development of plants was largely unknown at the start of this study. Our results revealed the primary function of ferritin in plants: although ferritins are not an essential iron source for development, they play a significant role in the defence machinery against oxidative stress.

Is ferritin a major site of iron storage in seeds?

FER2 is the only ferritin subunit accumulated in dry seeds, and it is degraded during germination. In *fer2*, the total iron content was not altered compared to wild-type. Iron contained in ferritin was estimated to represent no more than 5% of the total seed iron. Thus, it appears that ferritin does not constitute the major iron-storage form in Arabidopsis seed. Furthermore, iron stored into ferritin is not essential for development of the seedling, even under iron-limiting conditions. These results are consistent with findings that the vacuole is required for iron distribution in seeds and for re-mobilization of iron during germination through the activities of vacuolar NRAMP3 and NRAMP4 efflux transporters and the VIT1 influx transporter (Kim *et al.*, 2006; Lanquar *et al.*, 2005). These conclusions differ from those of studies on the importance of ferritins in pea seeds (Briat and Lobréaux, 1997; Briat *et al.*, 1999; Lobréaux and Briat, 1991). The amount of iron stored inside pea ferritins was estimated to be 92% of the total seed iron content in the embryo axis (Marentes and Grusak, 1998), a reason to consider this

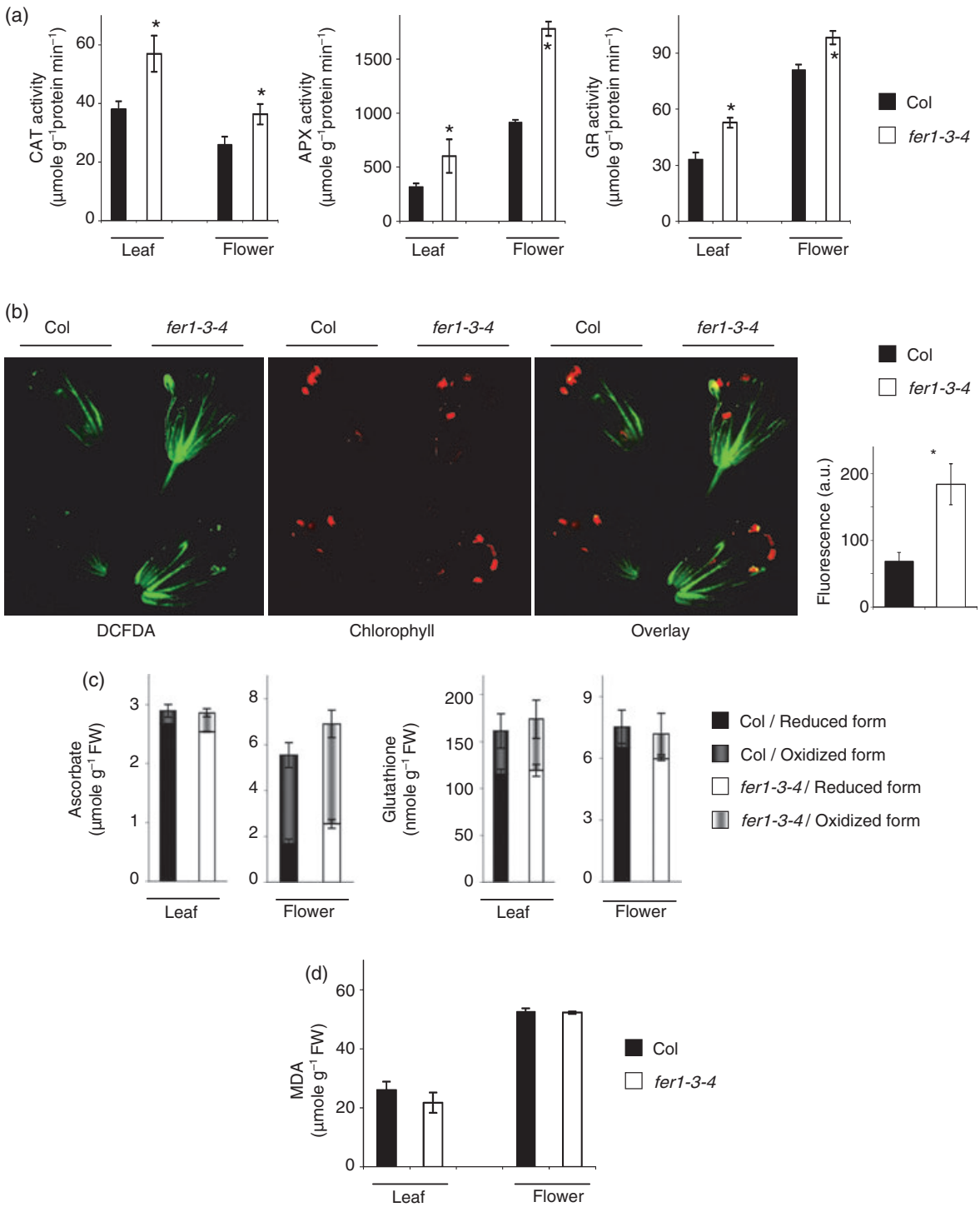


Figure 8. ROS-scavenging mechanisms are enhanced in *fer1-3-4* irrigated with water.
(a) ROS-detoxifying enzyme activities are enhanced in *fer1-3-4*. Leaves and flowers were harvested from plants irrigated with water, and assayed for catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) activities on total protein extracts. Values are means ± SD (n = 6). Significant differences between the two genotypes (P < 0.005) are indicated by asterisks.
(b) ROS over-accumulate in *fer1-3-4* flowers. Flower buds were collected from 4-week-old plants irrigated with water, and stained with DCFDA. Left panel, green fluorescence corresponding to ROS. Middle panel, red fluorescence resulting from chlorophyll fluorescence. Right panel, overlay. Quantification values are means ± SD (n = 24). The values for the two genotypes were significantly different (P < 0.005).
(c) The redox status of ascorbate and glutathione is not affected in *fer1-3-4*. Leaves and flowers were collected as described in (a). Values are means ± SD (n = 6).
(d) ROS damage estimated by the level of malondialdehyde (MDA). Leaves and flowers were collected as described in (a). Lipid peroxidation was measured as the level of thiobarbituric acid-reactive substances. Values are means ± SD (n = 6).

protein as the major form of iron storage in seeds. Thus, the intracellular site for iron storage in seeds may differ between legumes, such as pea, and Arabidopsis. Ferritins have only a minor iron-storage function in Arabidopsis. However, *fer2* seeds were more sensitive to MV, indicating that dynamic changes in ferritin levels during seed maturation and germination are probably necessary to prevent deleterious production of ROS by free iron.

Roles of ferritins in leaves

Three ferritin isoforms are present in leaves: FER1, FER3 and FER4. To investigate the functions of ferritin in leaves, we produced a triple *fer1-3-4* mutant, entirely lacking in ferritins. Under water irrigation, the growth of Col and *fer1-3-4* was similar. When irrigated with iron, the increased biomass observed for Col indicates that this metal was a limiting factor, and that it was not toxic. By contrast, *fer1-3-4* growth was reduced, probably due to a decrease in CO_2 fixation.

In mature leaves, photosynthesis was not affected by the absence of ferritin. The observation that the electron flux through PSII was not different in the mutant and Col under iron or water irrigation suggests that the absence of ferritins in the triple mutant did not have a severe impact on the photosynthetic transport machinery. However, the decrease in CO_2 fixation observed suggests that the photosynthetic electron transfer chain was less efficiently used by the Calvin cycle enzymes in the absence of ferritins.

The leaf is a major sink for iron accumulation in plants, and chloroplasts contain 80% of the metal present in this organ (Shikanai *et al.*, 2003). Ferritin accumulates mainly in non-green plastids, and a low level of this protein is found in mature chloroplasts in which the photosynthetic process is active (Briat and Lobréaux, 1997; Lobréaux and Briat, 1991; Theil and Hase, 1993). These observations have led to the hypothesis that ferritin may be an iron source at early stages of leaf development for the synthesis of iron-containing proteins involved in photosynthesis. However, alteration of young leaf development was not observed in *fer1-3-4*. Our results demonstrate that ferritins are not necessary either for producing a functional chloroplast or for proper development of the leaf.

Role of ferritin in reproductive organs

Pleiotropic defects in flowers were observed when *fer1-3-4* was grown under iron irrigation, leading to a strong reduction of fertility. These alterations were associated with an increase in the total iron content in the flowers, and a decrease in the stem, suggesting an alteration of iron transport and allocation between these organs. Indeed, transcriptomic analysis of a set of potential iron-transporter genes revealed that expression of many of them was modified in *fer1-3-4* compared to Col when grown under elevated iron conditions. These deregulations may arise either from differences in iron content between the genotypes, or from the absence of ferritins in the mutant. Some genes were upregulated in the stem of the mutant although the amount of iron was lower (*YSL3*, *YSL5*, *NRamp6*, *ZIP4*), and some genes were downregulated in the flower of the mutant where the iron concentration was higher (*NRamp5* and *NRamp6*). This suggests that deregulation is probabaly related to the absence of ferritin in the mutant, rather than the iron status of the tissues. Consistent with this, some genes were coregulated in both organs of the mutant even though the flowers and stem show different iron status. The genes that were upregulated in both organs were *NRamp1* and *YSL5*. The genes that were downregulated in both organs were *IRT1*, *IRT2*, *YSL4*, *YSL7* and *NRamp3*. Of these genes, the function of IRT1, NRAMP3 and YSL3 has been established (Lanquar *et al.*, 2005; Vert *et al.*, 2002; Waters *et al.*, 2006). Expression of the plasma membrane-located transporter IRT1 has been shown to be strongly repressed by iron (Vert *et al.*, 2002). NRAMP3, located on the tonoplast, has been shown to be essential for iron release from the vacuole to the cytosol (Lanquar *et al.*, 2005). Downregulation of these two genes in the mutant may function to limit iron toxicity by decreasing iron influx into the cell (by IRT1) and iron efflux from the vacuole (by NRAMP3). The downregulation of *YSL3* in the stem of the mutant could lead to a limitation of transport of iron–nicotianamine complexes to the flowers, where the iron excess could be toxic. The lack of functional characterization of most of the genes studied limits understanding of the deregulation patterns observed in the absence of ferritins. Nevertheless, this transcriptomic analysis made possible to select a small set of target genes and investigate their roles in iron transport in reproductive organs. Our results show that the absence of ferritin in reproductive organs strongly alters iron homeostasis and deregulates iron transport between organs, leading to alterations in fruit development, probably due to toxicity of the excess iron.

Iron, ferritins and ROS

The relationship between iron homeostasis and oxidative stress has been demonstrated in bacteria, yeast and animals, where the activity of key factors regulating iron homeostasis has been shown to be modulated by oxidative stress (Hantke, 2001; Kaplan *et al.*, 2006; Rouault and Klausner, 1996; Theil, 2007; Toledano *et al.*, 2007; Touati, 2000).

In plants, a link between iron homeostasis and oxidative stress has been suggested by physiological and molecular studies. Excess iron, or a mutation leading to an abnormally high level of iron, increases oxidative stress responses (Kampfenkel *et al.*, 1995; Pekker *et al.*, 2002; Pich *et al.*, 1994). Cytosolic *APX1* mRNA was shown to accumulate in response to iron (Fourcroy *et al.*, 2004). Pro-oxidants such as

NO, H_2O_2 and ozone, as well as high light intensity, induce ferritin synthesis (Arnaud *et al.*, 2006; Gaymard *et al.*, 1996; Lobréaux *et al.*, 1995; Murgia *et al.*, 2001, 2002; Savino *et al.*, 1997; Tarantino *et al.*, 2003). These reports suggest interactions between iron and ROS production in plants, but a direct link has not been established.

Our data show that ferritins play an essential role in avoidance of iron-dependent oxidative stress. In seeds, the absence of ferritin led to higher sensitivity to the pro-oxidant compound MV. In leaves and flowers, we show that, under normal plant development conditions (i.e. irrigation with water), the absence of ferritins in *fer1-3-4* led to increased activity of several ROS-detoxifying enzymes and enhanced ROS production. Nevertheless, the oxidized and reduced ascorbate and glutathione pools in the mutant were similar to those for Col, and ROS-associated damage was avoided, as determined by measuring lipid peroxidation. These results indicate that, under water irrigation, *fer1-3-4* is able to compensate for and bypass the lack of safe iron storage in ferritins by increasing the capacity of ROS-detoxifying mechanisms. However, when the plants were irrigated with high amounts of iron, the lack of ferritins in *fer1-3-4* led to pleiotropic defects both in vegetative and reproductive organs, which strongly impaired plant growth and fertility. It appears that, under high-iron conditions, the free-iron-associated ROS production overwhelmed the scavenging mechanisms in *fer1-3-4*. Our results show that ferritin function is essential for iron sequestration to avoid oxidative stress.

In animals, the primary function of ferritins inside cells is to store a pool of iron that is thus available for metabolism (Theil, 2003). A secondary function involves detoxification of labile iron and protection against ROS production (reviewed by Mackenzie *et al.*, 2008). By contrast, in bacteria, the primary function of ferritin is related to detoxification of iron and oxygen (reviewed by Carrondo, 2003). Our work demonstrates that the function of plant ferritin is closely related to those of bacteria. A recent report showed that, in *Chlamydomonas*, ferritin plays a role in protection of the cell under photo-oxidative stress conditions (Busch *et al.*, 2008). Thus, ferritin is an essential regulator of iron homeostasis and plays a key role in the control of iron-induced oxidative stress.

In plants, ferritin gene expression is modulated by many environmental factors including drought, cold, light intensity and pathogen attack. Throughout their life cycle, plants often experience these stresses, which are variable in intensity, location and duration, and which can transiently increase intracellular free-iron pools, leading to increased reactivity with oxygen to generate ROS. It is very likely that plant ferritins, by buffering iron, help plants to cope with adverse situations, the deleterious effects of which would be exacerbated if no system had evolved to take care of free reactive iron.

Experimental procedures

Plant materials

Arabidopsis thaliana L. (Columbia ecotype) plants were used for all experiments. The T-DNA insertion line *fer1-1* (SALK_055487) has been described previously (Dellagi *et al.*, 2005). *fer2-1* and *fer4-1* were provided by the Salk Institute (SALK_002947 and SALK_068629, respectively) and *fer3-1* was provided by the GABI program (GABI-KAT_496A08). To facilitate reading, the names of the mutants were simplified as follows: the simple mutants are named *fer1*, *fer2*, *fer3* and *fer4*, the double mutants *fer1-3*, *fer1-4* and *fer3-4* correspond to *fer1-1 fer3-1*, *fer1-1 fer1-4* and *fer3-1 fer4-1*, respectively, and the triple mutant *fer1-1 fer3-1 fer4-1* is named *fer1-3-4*. Simple mutants were back-crossed, and homozygous plants were selected by PCR screening using specific primers (Figure S1). *fer1* was crossed with *fer3* and with *fer4* mutants to generate the double mutants *fer1-3* and *fer1-4*. The cross between *fer1-3* and *fer1-4* enabled us to isolate the triple homozygous *fer1-3-4* mutant.

For complementation, the full-length *AtFer1* cDNA was cloned downstream of the 35S promoter in pCAMBIA 1301 (http://www.cambia.org) between the *Nco*I and *Nhe*I sites. The resulting plasmid was transferred to the GV3101 *Agrobacterium* strain (Koncz and Shell, 1986). Transformation of Col and *fer1-3-4* was performed using the floral dip procedure (Clough and Bent, 1998). Transformed plants were screened *in vitro* on a Murashige and Skoog standard medium (Sigma M5519, http://www.sigmaaldrich.com/) supplemented with 30 mg l^{-1} hygromycin. Three independent homozygous lines were selected for further analysis. For *fer2* complementation, a 35S::*AtFer1* Col line was crossed with the *fer2* mutant.

Plant growth conditions

Plants were sown on soil (Neuhaus Humin Substrat N2, Klasman-Deilmann GmbH, Geeste, Germany) in pots as single plant. Plants were grown in a controlled growth chamber at 23°C with a relative humidity of 70%, under short-day conditions (8 h light/16 h dark photoperiod) at a light intensity of 250 μmol m^{-2} sec^{-1}. Plants were irrigated as required (usually once a week) with water or an Fe-EDDHA solution (Sequestrene, http://www.fertiligene.com) at various concentrations. Grafting was performed as described previously (Rhee and Somerville, 1995) when the stalks were approximately 5–10 cm high.

Germination assays

All the wild-type and *fer2* seed pools used for germination analysis were obtained from 12 plants grown and harvested under the same conditions. Seeds were first stratified for 1 week in a controlled storage chamber at 4°C with a relative humidity of 30% under darkness. Then seeds were sown on de-ionized water agar medium (6 g l^{-1}) with or without methylviologen (MV; Sigma-Aldrich). Plates were placed at 4°C in darkness for 2 days, then transferred to a growth chamber at 23°C under long-day conditions (16 h light/8 h dark photoperiod) at 120 μmol m^{-2} sec^{-1}. The efficiency of germination was evaluated as radicle protrusion.

Quantitative RT-PCR

Total RNA was extracted using Trizol reagent (Euromedex, http://www.euromedex.com) according to the manufacturer's recom-

mendations. cDNA synthesis, quality control and quantitative RT-PCR were performed in a Light Cycler® (Roche Diagnostics, http://www.rochediagnostics.fr) according to the method described by Girin *et al.* (2007). PCR amplification of the various genes analyzed was performed using the 3′ UTR gene-specific primers listed in Table S1. The quantitative RT-PCR results were analyzed using Light-Cycler 3 data-analysis software (Roche). Relative transcript levels (RTL) were evaluated by calculation of the difference between the crossing time of the target gene and the threshold crossing time of the control gene for the respective templates, thus normalizing the target gene expression to the control gene expression (Arrivault *et al.*, 2006). Gene expression was monitored in triplicate, and results were standardized using the *SAND* gene (Czechowski *et al.*, 2005; Remans *et al.*, 2008).

Immunodetection of ferritins

Total protein extracts were prepared from samples as described previously (Lobréaux *et al.*, 1992). Immunodetection of ferritin was performed using a rabbit polyclonal antiserum raised against purified FER1 protein (Arnaud *et al.*, 2007). To monitor ferritin content by Western blotting in the various mutants used in this study, the specificity of the serum directed against the FER1 subunit towards the four Arabidopsis ferritin subunits was determined. The four recombinant ferritin subunits were produced in *E. coli*, and the serum was used in Western blots to detect serial dilutions of the four purified recombinant proteins. Qualitatively, it reacted with all four subunits. Quantitatively, the detection was equivalent for FER1, FER2 and FER3, but was about 10 times lower for FER4.

Iron content measurement

Measurement of total iron content was performed as previously described (Lobréaux and Briat, 1991). Samples were mineralized as described by Beinert (1978). Iron concentration was measured by absorbance of Fe^{2+}-bathophenanthroline disulfonic acid (BPDS) (1%) at 535 nm using thioglycolic acid (Sigma) as the reducing agent. Determination of iron content was performed using a range of standard Fe solutions (Carlo Erba, http://www.carloerbareagenti.com).

Determination of CO_2 fixation and PSII efficiency

Measurements were made on attached fully expanded leaves of 5-week-old plants grown on soil, and irrigated with water or 2 mM Fe-EDDHA, using a portable CIRAS-2+ CFM system (PP Systems, http://www.ppsystems.com), which allows the environmental conditions inside a 4 mm cuvette to be precisely controlled. Response curves to CO_2 fixation were obtained at various light intensities (photosynthetically active radiation from 80 to 1000 μmol photons m^{-2} sec^{-1}). ϕ_{PSII} was determined as described previously (Genty *et al.*, 1989).

Imaging of ROS production

ROS imaging in flower buds was performed using 5-(and-6)-carboxy-2′,7′-dichlorodihydro-fluorescein diacetate (DCFDA, Molecular Probes, http://www.probes.com) as described by Hauser *et al.* (2006). All fluorescence images were obtained using a macro-zoom Olympus MVX10 microscope (http://www.olympus.fr) using the 1× objective and constant exposure times and a Hamamatsu Orca camera (http://www.hamamatsu.com), and were analyzed using the Cell-P soft-imaging system. Quantification was performed using ImageJ software (http://rsbweb.nih.gov).

Enzyme activities and metabolite measurements

Samples (0.4–0.6 g of tissues) were extracted in 1 ml of 50 mM KH_2PO_4 pH 7.6, 1 mM EDTA, 0.1% v/v Triton X-100 and 1 mM PMSF (plus 5 mM ascorbate in extracts used for APX activity). The homogenate was centrifuged three times at 15 000 ***g*** for 10 min each, and the supernatant was used for enzyme analysis. All enzymes were assayed at 25°C in a 1 ml reaction mixture on a Hitachi U-2800 spectrophotometer (http://www.hitachi.fr). Catalase and ascorbate peroxidase activities were measured as described previously (Cakmak and Marschner, 1992), and glutathione reductase activity was assayed according to the method of Hodges and Forney (2000). L-ascorbic acid (AsA), dehydroascorbate (DHA), reduced glutathione (GSH) and oxidized glutathione (GSSG) were quantified as described by Kampfenkel *et al.* (1995). Estimation of lipid peroxidation was assessed using the thiobarbituric acid-malondialdhyde (TBA-MDA) assay method as modified by Hodges *et al.* (1999).

Acknowledgements

This work was funded by Institut National de la Recherche Agronomique and Centre National de la Recherche Scientifique, the Action Concertée Incitative 'Biologie Cellulaire Moléculaire et Structurale' (grant number BCMS166) from the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche, and by the ANR-Blanche DISTRIMET (grant number 25383) from the Agence Nationale de la Recherche. K.R. was supported by a thesis fellowship from the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche. We thank the GABI program and the Salk Institute Genomic Analysis Laboratory (SIGNAL) for providing the sequence-indexed Arabidopsis T-DNA insertion mutants, and the Nottingham Arabidopsis Stock Centre (NASC) for providing seeds. We acknowledge Myriam Dauzat (LEPSE, INRA Montpellier) for her help in photosynthesis and chlorophyll fluorescence measurements. We are very grateful Dr Elizabeth Pilon-Smits, Dr Marinus Pilon (Department of Biology, Colorado State University, CO, USA) and Dr Marc Lepetit for helpful discussions, and critical reading of the manuscript. This work is dedicated to Tam and Lo.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Additional experimental procedures.

Appendix S2. Data used for determination of expression values from quantitative RT-PCR in three biological replicates.

Figure S1. Isolation and molecular characterization of ferritin-less mutants.

Figure S2. Kinetic of germination of Col and *fer2* mutant seeds.

Figure S3. Photosynthetic complexes and chlorophyll contents in the *fer1-3-4* mutant.

Figure S4. Tissue localization of *AtFer1*, *AtFer3* and *AtFer4* expression in flowers, and iron accumulation in Col and *fer1-3-4* flowers.

Table S1. Primer sequences used in quantitative RT-PCR experiments and PCR efficiencies.

Table S2. RT-PCR data.

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