Analysis of Arabidopsis thaliana atfer4-1, atfh and atfer4-1/atfh mutants uncovers frataxin and ferritin contributions to leaf ionome homeostasis

Abstract

Ferritins are iron-storage proteins involved in the environmental and developmental control of the free iron pool within cells. Plant ferritins are targeted to mitochondria as well as to chloroplasts. AtFer4 is the *Arabidopsis thaliana* ferritin isoform that can be also targeted to mitochondria. Frataxin is a mitochondrial protein whose role is essential for plants; lack of AtFH frataxin causes early embryo-lethality in *Arabidopsis*. Because of that, the *Arabidopsis atfh* KO mutant is propagated in heterozygosis.

For exploring the functional interaction between frataxin and ferritin, *Arabidopsis* double mutant *atfer4-1/atfh* was isolated and its physiological parameters were measured, as well as its ionome profile, together with those of both *atfer4* and *atfh* single mutants, in different conditions of Fe supply.

Impairment of both ferritin and frataxin did not lead to any effect on mitochondrial respiration. However, ionomics revealed that the content of macro- and microelements, occurring when the nutritional Fe supply changes, were altered in the mutants analysed. These results suggest that both ferritin and frataxin can contribute to the composition of the leaf ionome and also confirm ionomics as an excellent tool for detecting alterations in the plant's physiology.

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Keywords

Arabidopsis
Ferritin
Frataxin
Ionomics
Iron homeostasis
Mitochondria
Molybdenum

1. Introduction

Iron (Fe) is an essential element for plants: it forms part of the prosthetic groups of different proteins directly involved in photosynthesis and respiration (as heme or iron–sulfur [Fe–S] clusters) (Balk and Schaedler, 2014; Couturier et al., 2013) as well as in other key metabolic pathways for plants, such as nitrogen assimilation and scavenging of Reactive Oxygen Species (ROS). Beside its role in Fe-containing proteins, Fe is also involved, as nitrosyl and dinitrosyl Fe complexes, in various signaling transduction pathways (Ramirez et al., 2011). Because of the relevance of Fe in the various house-keeping functions of plants, the processes of its uptake from soil to the roots, the transport from roots to the various plant organs, its storage in the various sink organs and trafficking within cells of the various sink organs, are finely regulated by a tight homeostatic control (Kobayashi and Nishizawa, 2012; Grillet et al.,2013).

Ferritin is an iron-storage protein present in both animal and plant cells, as a 24-mer cage-like structure able to sequester or release Fe upon demand. The biochemical and physiological properties of such cage-like iron-reservoir (i.e. the oxidation of Fe(II) to Fe (III) by a ferritin ferroxidase center and its deposition, in mineral form, inside the ferritin cavity) have been elucidated (Theil et al., 2006; Arosio et al., 2009).

Despite ferritins from animal and plants sharing several common characteristics, some features are distinctive for plant ferritins (Briat et al., 2010). The role of plant ferritin as a protectant against oxidative stress (Ravet et al., 2009; Sudre et al., 2013), its environmental and developmental regulation (Tarantino et al., 2003; Murgia et al., 2007) and the signal transduction pathways leading to its accumulation have been studied (Murgia et al., 2002, 2004; Arnaud et al., 2006).

Animal ferritins are localized in the cytoplasm, as well as in mitochondria; the physiological relevance of ferritin localization in such organelles has been investigated in detail (Arosio and Levi, 2010). Plant ferritins, beside their prominent localization in chloroplasts, can also be localized in mitochondria (Zancani et al., 2004; Tarantino et al., 2010a; Vigani et al., 2013). It has been demonstrated that ferritin localization in mitochondria from cucumber roots is functional, i.e. the 24-mer formed by ferritin truly acts as an Fe store (Vigani et al., 2013).

Frataxin is a protein essential for mitochondrial functionality in humans: its deficiency causes a severe neurodegenerative disease named Friedreich's ataxia (Taroni and DiDonato, 2004). Frataxin is present in bacteria, unicellular eukaryotes, animal cells (Foury et al., 2007; Vasquez-Manrique et al., 2006) and plants (Busi et al., 2004; Vazzola et al., 2007; Murgia et al., 2009). Frataxin has an essential role, in animal as well as in plant cells: lack of frataxin causes early embryo lethality both in mice (Sarsero et al., 2004) and in *Arabidopsis* (Vazzola et al., 2007).

Notably, an *Arabidopsis* frataxin knock-down mutant with 50% frataxin reduction shows impaired activity of mitochondrial enzymes containing Fe–S clusters (Busi et al., 2006); nitric oxide (NO) accumulates in the roots of such an *Arabidopsis* frataxin knock-down mutant, leading to the hypothesis that NO is required to counteract the iron-mediated oxidative stress induced by reduction of frataxin (Martin et al., 2009).

Because of the impact of frataxin impairment on human health, its primary physiological role has been the object of intense investigation in different model systems, and the different results have been matter of scientific discussion for many years. Different functions have been suggested for frataxin, which are directly or indirectly involved in the control of mitochondrial Fe homeostasis. The two main proposed functions are: i) mitochondrial iron storage/detoxification; ii) Fe-chaperone, attributed because of its role in biosynthesis of [Fe–S] clusters and heme groups; for detailed reviews on the various proposed roles, see Murgia et al. (2009) and Jain and Connolly (2013).

Certainly frataxin and ferritin are molecular partners keeping Fe under control, in plants (Ramirez et al., 2011). In humans, a protective role in Friedreich's ataxia has been proposed for mitochondrial ferritin (Campanella et al., 2008). However, their functional interaction(s) have not yet been explored in detail, in plants.

The goal of the present work is to unveil the functional link between ferritin and frataxin in plant mitochondria. *Arabidopsis* possesses 4 different ferritin isoforms (AtFer1-4) and AtFer4 is the only one with documented involvement in mitochondrial Fe homeostasis: it can also be targeted in mitochondria of green leaves (Tarantino et al., 2010a) and its mutation, in heterotrophic cells, alters Fe homeostasis resulting in a change in mitochondrial Fe trafficking (Tarantino et al., 2010b).

We therefore isolated an *Arabidopsis atfer4-1/atfh* double mutant with impaired ferritin AtFer4 and frataxin AtFH; we investigated its physiological parameters (photosynthesis and respiration) and we profiled its leaf ionome (macro- and microelements), together with that of the single *atfer4-1* and *atfh* mutants, in order to discover any interactions between frataxin and ferritin. Ionomics has been defined as the study of the social network of mineral nutrients in a given tissue (Baxter, 2009) and it has been demonstrated to be an excellent tool for investigating changes in the physiological plant status, associated with the changes in Fe nutritional status (Baxter et al., 2008).

2. Material and methods

2.1. Plant material

Arabidopsis plants were grown in a greenhouse at 25 °C, 8 h/16 h light/dark photoperiod, at 100 μE/m^2 sec, into Arabaskets (Beta Tech) on Technic n.1 DueEmme soil (Netherlands) and constantly watered with either tap water (controls) or tap water supplemented with 0.1% Fe-EDDHA, BasaferPlus®.

2.2. Isolation of *atfer4-1/atfh* mutant

Progeny plants grown from seeds collected from an heterozygous *atfh* KO plant, were classified by PCR, with Fxfor-Fxrev primer pair annealing respectively, in the first and in the last exon of AtFH gene, for identifying the wt AtFH allele, and with Fxfor-LBa1 for identifying the mutated AtFH allele bearing T-DNA insertion, as already reported in Vazzola et al. (2007). Heterozygous *atfh* KO plants were crossed with *atfer4-1* mutants (Tarantino et al., 2010a). F2 (and successive) progeny plants were classified, by using Fxfor-Fxrev and Fxfor-LBa1 primer pairs, as indicated above, as well as dAT4-rAT4 and dAT4-LBA1 primer pairs, which respectively amplify native and mutated AtFer4 allele bearing the T-DNA insertion, as described in Tarantino et al. (2010a).

Fxfor, Fxrev and LBa1 primer sequences and amplification conditions are reported in Vazzola et al. (2007). The rAT4 sequence is 5′-CGCACTCGTCTGAGTACTTTTGT-3'; for the amplification of the AtFer4 native gene fragment (~1000 bp), a PCR reaction with dAT4-rAT4 primer pair, 55 °C anneal. Temp. with 2 mM $MgCl_2$ is performed. dAT4 primer sequence and amplification conditions for dAT4-LBA1 primer pair are in Tarantino et al. (2010a). For RT-PCR analysis, the AtFH transcript was amplified according to Vazzola et al. (2007) whereas TUB4 was amplified according to Tarantino et al. (2005).

2.3. Siliques inspection

Siliques were detached and immersed in a clearing solution and then inspected at the microscope as described in Vazzola et al. (2007).

2.4. Measurements of O_2 evolution and consumption

Oxygen consumption and evolution were measured in rosette leaves detached from 3 to 4 weeks old plants, by using the Clark-type oxygen electrode (Hansatech Ltd, King's Lynn, Norfolk, U.K.) as described in Tarantino et al. (2010a). Rates were analysed with Oxylab V1.15 software. Leaves were then removed from the chamber and put in a vial containing 1–3 ml dimethylformamide for chlorophyll extraction and quantification, according to Tarantino et al. (2005).

2.5. Ionomics

Leaves of four week old wt Col, *atfer4-1*, *atfh* and *atfer4-1/atfh* plants grown in control or +Fe soil, were cut at the petiole and thoroughly rinsed in distilled water; water on the leaf surface was then gently removed with absorbing paper and leaves were put in calibrated 15 ml tubes (5 leaves/tube). Concentration of various elements was then measured by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) according to Vigani et al. (2013). Statistical analysis was carried out by using Duncan's test with SPSS software. PCA analysis was performed by using the MetaGeneAlyse platform at http://metagenealyse.mpimp-golm.mpg.de (Daub et al., 2003). Data for the PCA are median centered and $\log_{10}$ transformed.

3. Results

3.1. Isolation of the double mutant *atfer4-1/atfh*

Lack of frataxin is embryo lethal in *Arabidopsis* and the homozygous *atfh* KO mutant dies early during embryogenesis (Vazzola et al., 2007); the *atfh* mutant is therefore propagated in heterozygosis. As expected, expression of the AtFH gene is reduced in the *atfh* mutant (Fig. S1).

The *Arabidopsis atfh* mutant has been crossed with the *atfer4-1* mutant (Ravet et al., 2009; Tarantino et al., 2010a) and 91 F2 single progeny plants have been tested, one by one, by PCR; the genetic class bearing both mutations in homozygosis could not be recovered, whereas mutant plants homozygous for *atfer4-1* KO allele and heterozygous for *atfh* KO allele (named from now onwards *atfer4-1/atfh* for simplicity) have been identified and propagated further. Representative results of such PCR screenings leading to the identification of *atfer4-1/atfh* plants, are shown in Fig. S2.

As already observed for the single homozygous *atfh* mutant (Vazzola et al., 2007), the knocking-out of the AtFH gene also causes embryo-lethality in the *atfer4-1* genetic background; lack of the above indicated genotypic class in F2 progeny plants cannot be attributed to events which either precede or follow embryogenesis, such as the reduced fertility of gametes bearing both mutations or the decreased viability of putative mature double homozygous *atfer4-1atfh* seeds. Indeed:

a)

The siliques produced by the *atfer4-1/atfh* mutant, when inspected at the stereomicroscope show that some seeds are dramatically reduced in size leaving therefore a “hole”, corresponding to aborted embryos (Fig. 1A), as already described for *atfh* (Vazzola et al., 2007).

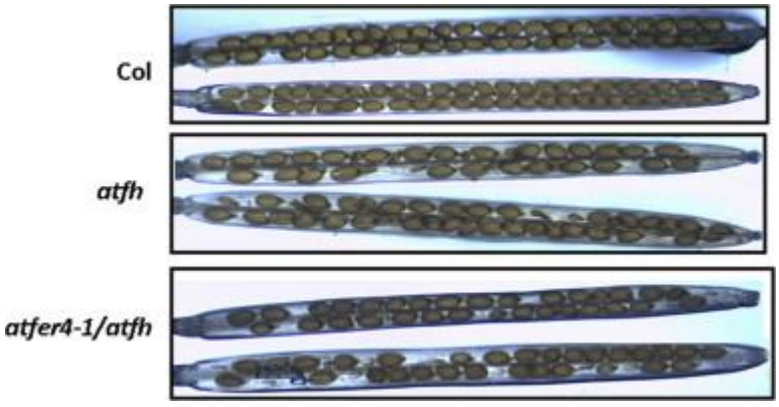

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Fig. 1. Progeny plants of wt Col, *atfh* and *atfer4-1/atfh* mutants, grown under control condition, have been first classified by PCR, allowed to produce seeds and their siliques cleared and inspected at the stereomicroscope.

b)

Out of 91 F2 single progeny plants tested, 12 individuals were homozygous for the *atfer4-1* mutant allele and heterozygous for the *atfh* mutant allele (the expected number of such a genotype is 11.3, i.e. 2/16 of 91 individuals); such correspondence between the expected and observed numbers is consistent with embryo lethality of the double mutant bearing both mutations in homozygosis and not with infertility, or reduced fertility, of gametes bearing both mutant alleles; in this last case, indeed, the double mutant bearing the *atfer4-1* mutant allele in homozygosis and *atfh* mutant allele in heterozygosis would also be missing from the Fe segregation (in the case of infertility of both gametes) or would be present at reduced frequency compared with that observed (in the case of reduced fertility of either of the gametes).

c)

Mature F2 progeny seeds germinate and the corresponding seedlings thrive and reach maturity (Table S1).

Such a double mutant has been propagated by self-fertilization; for all the experiments described below, progeny plants from the *atfh* mutant as well as from the *atfer4-1/atfh* mutant have been first genotyped, one by one, by PCR, for the identification of plants with the proper genotype.

3.2. O_2 evolution and O_2 consumption in *atfer4-1*, *atfh* and *atfer4-1/atfh* mutant plants

Wild type (wt) Col, *atfer4-1*, *atfh* and *atfer4-1/atfh* mutants have been grown on control soil (Fe sufficiency) or on soil regularly supplemented with Fe (indicated from now onwards as +Fe) and no phenotypic differences could be observed between wt and mutant plants (Fig. 2A).

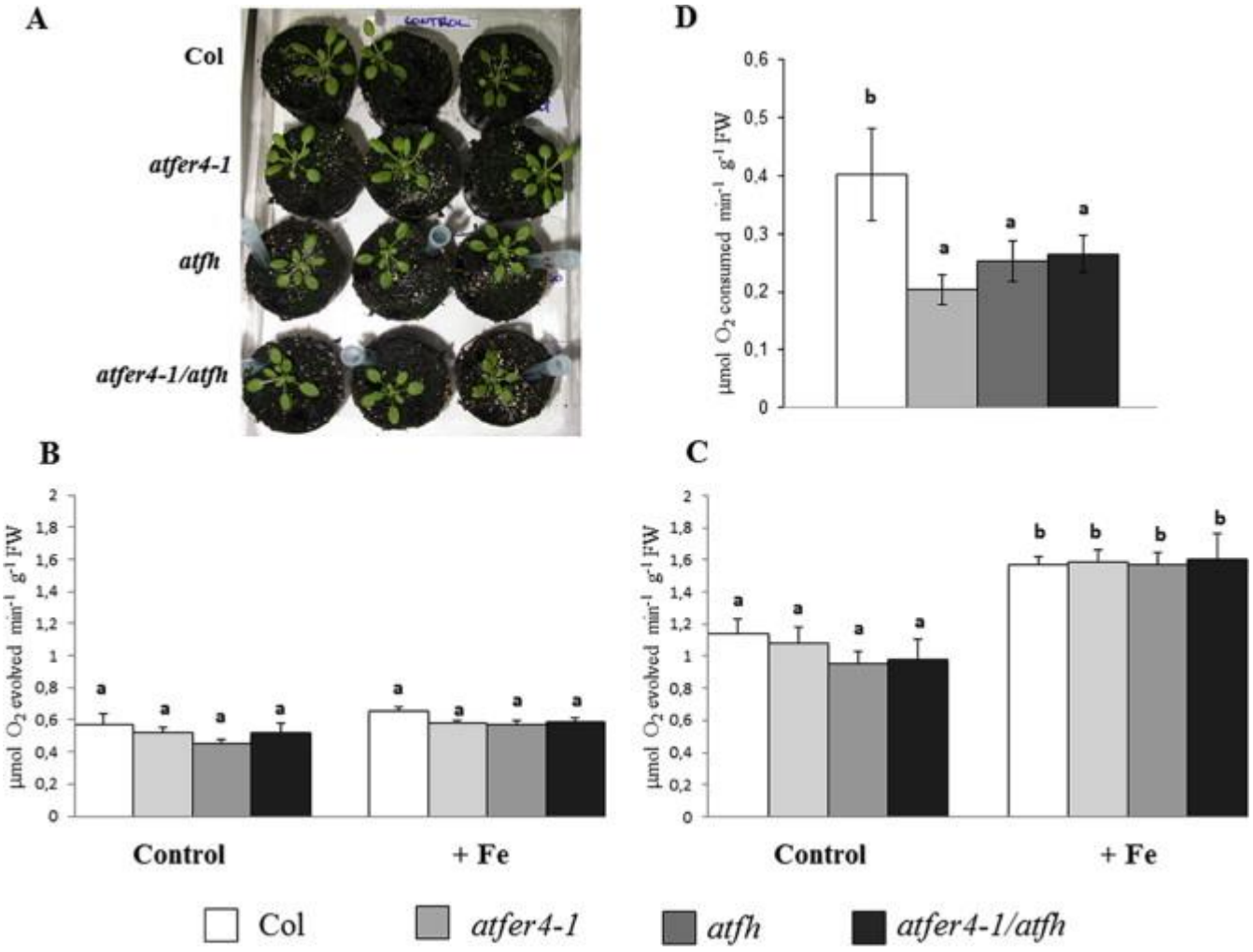

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Fig. 2. Four weeks old wt Col and *atfer4-1*, *atfh*, *atfer4-1/atfh* mutant plants. A Phenotype of Col, *atfer4-1*, *atfh*, *atfer4-1/atfh* plants grown in control soil. B Oxygen evolved in leaves of four weeks old plants grown in control or + Fe soil, when illuminated at 200 µE/m^2 sec. Values, expressed as µmol O_2 evolved/min mg chlorophyll, are the means ± SE of three independent samples, each consisting of five leaves detached from at least three different plants. C Oxygen evolved in leaves of four weeks old plants grown in control or + Fe soil, when illuminated at 800 µE/m^2 sec. Values, expressed as µmol O_2 evolved/min mg chlorophyll, are the means ± SE of three independent samples, each consisting of five leaves detached from at least three different plants. D Oxygen consumed in leaves of four weeks old plants grown in control soil, when illuminated at 200 µE/m^2 sec. Values, expressed as µmol O_2 consumed/min g fresh weight, are the means ± SE from nine independent samples, each consisting of five leaves detached from at least three different plants. Different letters indicate statistical significance, according to Duncan's test, with $p < 0.05$.

Photosynthetic O_2 evolution has been evaluated in rosette leaves, at two different light intensities, 200 µE/m^2 sec (Fig. 2B) or 800 µE/m^2 sec (Fig. 2C). Wt plants showed, as expected, an increase in O_2 evolution upon illumination from 200 to 800 µE/m^2sec, when grown both in control as well as in +Fe (Fig. 2B–C). Interestingly, wt plants grown under control or +Fe conditions showed, at 200 µE/m^2sec, the same O_2 evolution values (~0.6 µmol O_2 evolved/min mg chlorophyll) (Fig. 2B). Therefore, the Fe available to plants in the control soil fully meets the requirements of the photosynthetic apparatus, at least at light intensities close to the one used for growth, thus suggesting that the Fe supplemented in the +Fe soil exceeds plant requirements for photosynthesis

occurring at 200 μE/m^2sec. The same result has been also observed for chlorophyll, which does not increase in wt plants grown in +Fe, when compared to plants in control soil (Fig. S3).

However, under higher light intensity (800 μE/m^2sec), an increase in O_2 evolution is observed; such an increase, in wt plants grown in +Fe, is higher than the increase observed in wt plants grown in control soil (around 200% and 240% increase, respectively) (Fig. 2B–C). This result shows the positive contribution of the Fe supplementation in the +Fe soil, to the photosynthetic apparatus; indeed, plants in +Fe better meet the Fe demand due to the increased photosynthetic electron transfer occurring under higher light intensity.

The mutants *atfer4-1*, *atfh* and *atfer4-1/atfh* show the same values as their corresponding wt, in the two nutritional Fe conditions and at the two illumination intensities (Fig. 2B–C), indicating that impairment in either ferritin AtFer4 and/or in frataxin AtFH does not have any consequences in the leaf photosynthetic activity.

O_2 consumption is affected in the *atfer4-1* mutant, when grown in control condition (Fig. 2D), as already reported in Tarantino et al. (2010a) as well as in *atfh* and *atfer4-1/atfh*, whereas in +Fe, no differences are observed between wt and mutant lines (Fig. S4). Both ferritin and frataxin are involved in mitochondrial Fe trafficking, so the lack of differences in oxygen consumption in mutant plants grown at +Fe when compared to wild type is noteworthy. Excess Fe causes an increase in toxic free Fe ions, and therefore the perturbation of AtFH and AtFer4 genes is expected to have some effect on mitochondrial activity.

3.3. Ionomics on *atfer4-1*, *atfh* and *atfer4-1/atfh* leaves

Ionome profiling has been performed on leaves of 4 weeks old wt Col, *atfer4-1*, *atfh* and *atfer4-1/atfh* plants, grown in control or in +Fe (Figs. 3–4).

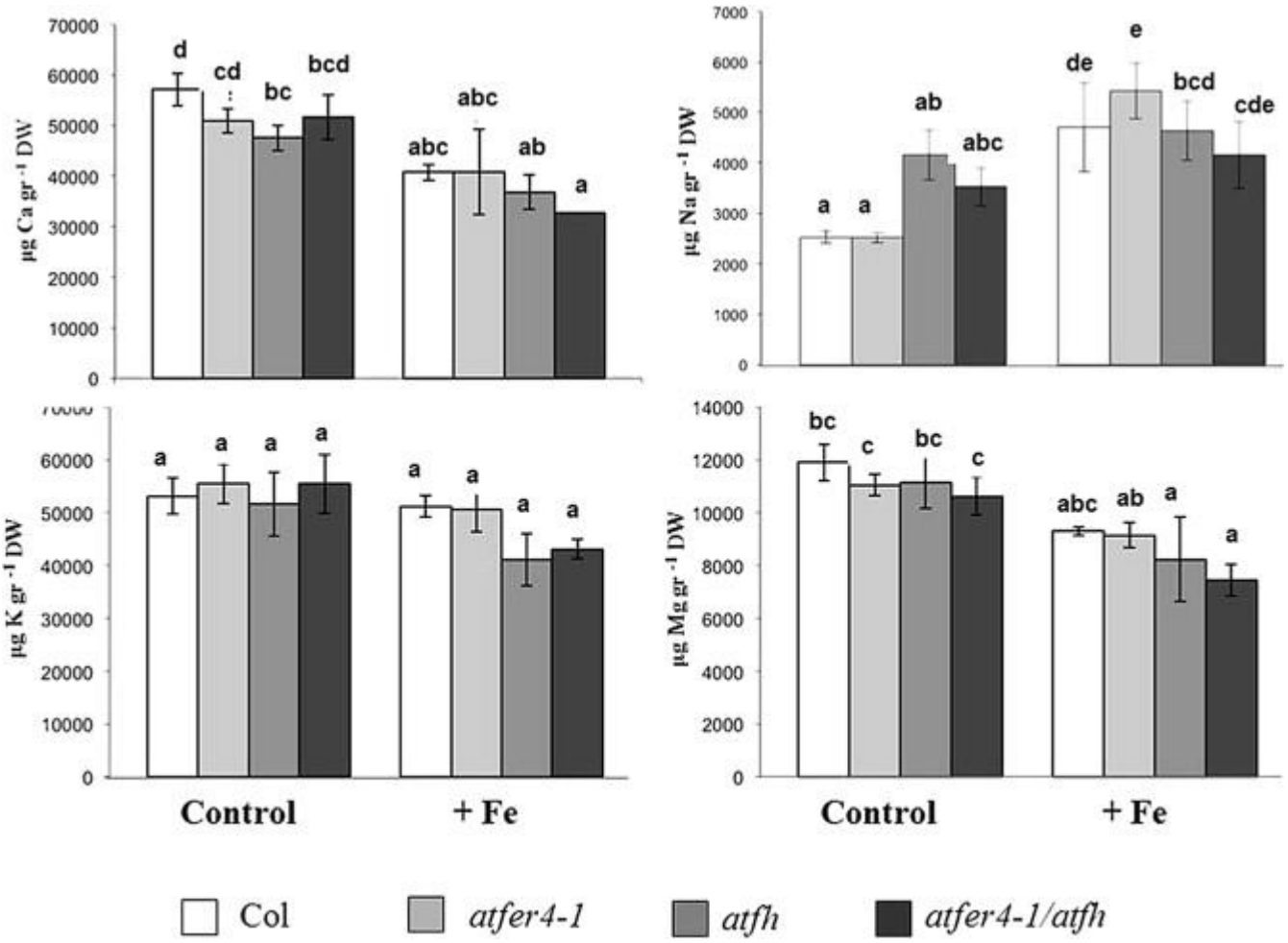

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Fig. 3. Concentration of the macroelements Na, Ca, K, Mg in leaves of four weeks old Col, *atfer4-1*, *atfh*, *atfer4-1/atfh* plants grown in control or +Fe soil. Values, expressed as µg/g dry weight, are the means ± SE of at least 5 independent samples, each consisting of 5 leaves detached from 3 to 5 different plants. Different letters indicate statistical significance, according to Duncan's test, with $p < 0.05$.

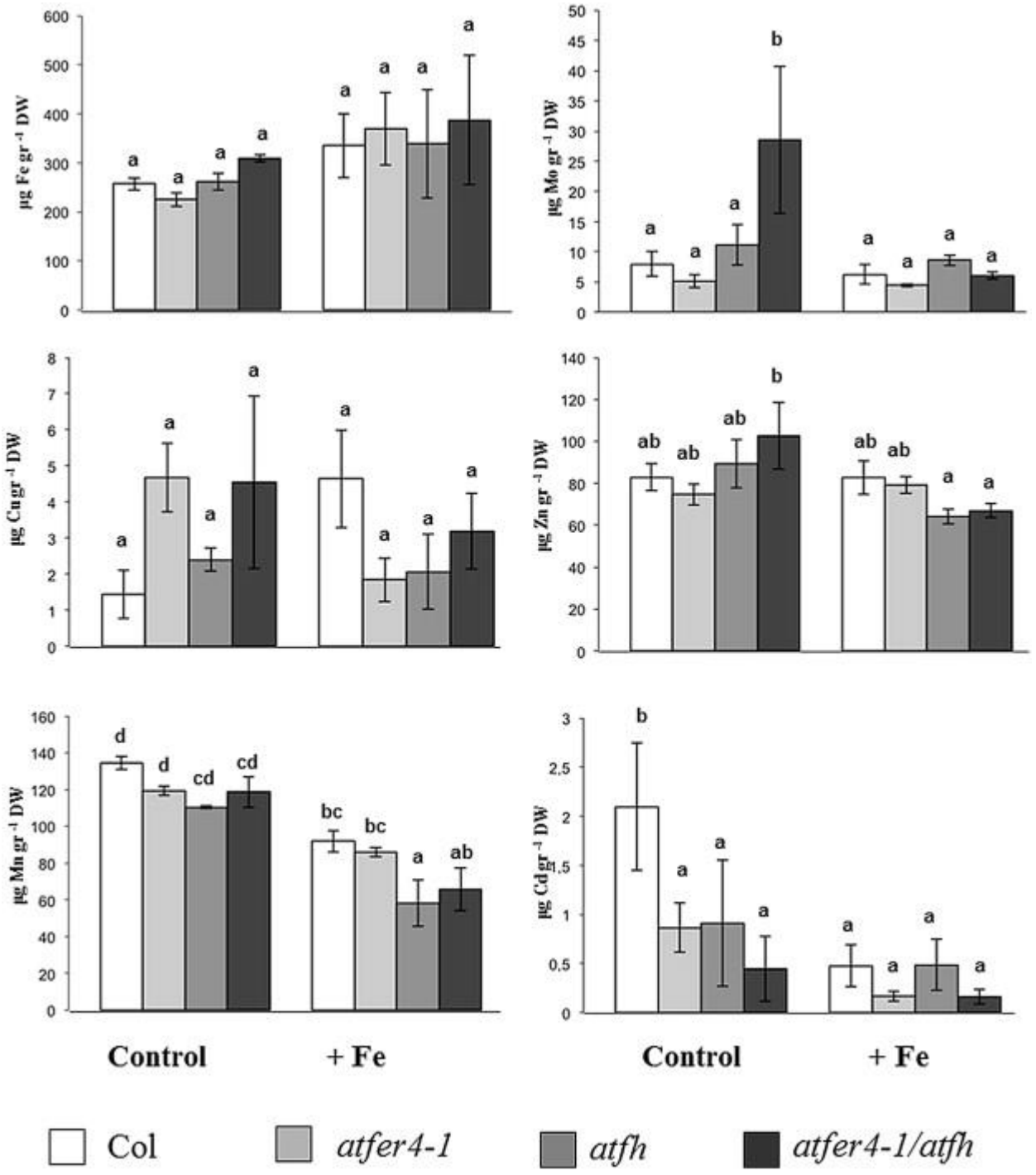

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Fig. 4. Concentration of the microelements Fe, Zn, Mn, Mo, Cu, Cd in leaves of four weeks old Col, *atfer4-1*, *atfh*, *atfer4-1/atfh* plants grown in control or +Fe soil. Values, expressed as µg/g dry weight, are the means ± SE of at least 5 independent samples, each consisting of 5 leaves detached from 3 to 5 different plants. Different letters indicate statistical significance, according to Duncan's test, with $p < 0.05$.

The concentration of various macro- and microelements, in wt Col plants, is dependent on the Fe availability, as observed for sodium (Na), calcium (Ca), magnesium (Mg) (Fig. 3) and for manganese (Mn) and cadmium (Cd) (Fig. 4): Na concentration increases, whereas Ca, Mg, Mn and Cd concentrations decrease in +Fe, when compared to control. The concentration of various macro-

and microelements are altered in the three *atfer4-1*, *atfh* and *atfer4-1/atfh* mutants, as reported in Figs. 3–4.

Such alterations can consist in a difference between genotypes in control condition or between genotypes in +Fe condition or even, for a given mutant, in an alteration of the fold concentration change in +Fe soil with respect to control soil, when compared to the fold change observed in the wt line. Fold concentration changes are reported in Table 1.

Table 1. Fold change of macro- and microelements concentrations, in leaves of four weeks old Col, *atfer4-1*, *atfh*, *atfer4-1/atfh* plants grown in +Fe soil with respect to growth in control soil (concentration in +Fe soil/concentration in control soil). Statistical significant ion concentration differences, between values in +Fe and control conditions are reported in bold.

	Ca	**K**	**Mg**	**Na**	**Cu**	**Fe**	**Zn**	**Mn**	**Mo**	**Cd**
Col	**0.712**	0.963	0.782	**1.864**	3.244	1.303	0.996	**0.684**	0.786	**0.228**
atfer4-1	0.802	0.913	**0.828**	**2.160**	0.395	1.642	1.058	**0.721**	0.857	0.193
atfh	0.774	0.795	**0.738**	1.115	0.122	1.296	0.718	**0.527**	0.772	0.533
atfer4-1/atfh	**0.634**	0.777	**0.700**	1.179	0.701	1.254	**0.652**	**0.554**	**0.213**	0.362

Under control condition, Ca concentration is lower in the *atfh* mutant, when compared to wt, while in +Fe condition, Ca concentration is similar among genotypes (Fig. 3). However, Ca concentration in +Fe condition decreases both in wt and in the *atfer4-1/atfh* mutant, when compared to control condition (Table 1).

Sodium is higher in *atfh* and *atfer4-1/atfh* mutants than in wt or in *atfer4-1*, when in control condition; nevertheless, neither *atfh* nor *atfer4-1/atfh* mutants show any further increase in Na concentration, when in +Fe, differently from what observed in the other lines (Fig. 3; Table 1).

Magnesium concentration significantly decreases, in all mutant plants when grown in +Fe, with respect to control condition (Fig. 3; Table 1).

Both potassium and copper concentrations are similar in wt and mutants plants, and do not change with +Fe treatment (Figs. 3–4).

Interestingly, Fe concentration does not increase in wt plants, when grown in +Fe; such result is consistent with the results shown in (Baxter et al., 2008), confirming that Fe concentration can be itself a poor indicator of Fe nutritional status, due to the strict Fe homeostatic control occurring in plant cells. Manganese concentration is dependent on Fe supply, as it diminishes in all +Fe plants (Fig. 4) and *atfh* and *atfer4-1/atfh* mutants grown in +Fe have lowest Mn concentrations. Cd concentration is lower, in control condition, in all tested mutants with respect to wt; in +Fe soil, Cd concentration in the mutants does not change with respect to control condition.

Most interestingly, molybdenum (Mo) concentration is higher in the *atfer4-1/atfh* mutant grown in control condition than in all other tested lines and decreases in +Fe condition to a value similar to those measured in the other lines in both treatments (Fig. 4). Last, in the *atfer4-1/atfh* double mutant zinc concentration is significantly lower in +Fe condition when compared to the control one and indeed the fold change is most pronounced in the *atfer4-1/atfh* double mutant (Fig. 4 and Table 1).

Results of the Principal Component Analysis (PCA) on the ionome data set (Figure S5) are not clearcut, since some clusters overlap; they nevertheless suggest that the variance of the ionomic

data might be mainly due to the different genotypes analysed, as explained in PC1 where the clusters at the extremes are the wt and the double mutant *atfer4-1/atfh*.

4. Discussion

Although frataxin is involved in keeping mitochondrial Fe under control (Murgia et al., 2009; Jain and Connolly, 2013) and ferritin is a functional Fe-store in mitochondria (Vigani et al., 2013), their role is still not well defined in plant cells, nor their putative functional interaction (Ramirez et al., 2011). For addressing such issues we isolated and analysed the *Arabidopsis* double mutant *atfer4-1/atfh*. Ionomics analysis on *atfer4-1*, *atfh* and *atfer4-1/atfh* mutants revealed changes in the mineral fingerprint of the double mutant *atfer4-1/atfh*, involving at least Mo and Zn, with respect to wt or the two single mutants; these results suggest a contribution of both AtFH and AtFer4 in the cellular ionome profiling.

Ionomics has been defined as the study of the social network of mineral nutrients in a given tissue (Baxter, 2009). Iron is an essential element, however its potential toxicity requires a tight control of its homeostasis, which justifies the low changes in shoot Fe concentration in *Arabidopsis* plants grown with a 10 fold change of Fe availability (Fe sufficiency versus Fe deficiency) (Baxter et al., 2008). However, ionomics applied on the same analysed material uncovered clear changes in concentrations of other elements, such as Mn, Co, Zn, Mo and Cd, which have been suggested as signatures associated with Fe nutritional status (Baxter et al., 2008).

We could confirm that Fe concentration (and chlorophyll) did not increase in leaves of wt *Arabidopsis* plants grown with constant Fe excess, when compared with control growth, consistently with the observations that Fe concentration in the plant can be a poor marker of Fe nutritional status itself, due to its tight homeostatic control (Baxter et al., 2008). Although no change in Fe concentration is observed in wt plants grown in +Fe soil, such plants evolve more O_2, when exposed to high light intensity, than plants grown in control soil. Notably, the reduction of O_2 consumption observed in all the mutants grown in control soil, when compared to wt, is not observed any longer when mutants are grown in +Fe. To clarify such a point it will be interesting, in the future, to measure the non-respiratory fraction of O_2 consumption in mutants grown in control and in +Fe; indeed, it is known that non-respiratory O_2 consumption is altered, in the presence of excess Fe (Vigani et al., 2013). The differences observed in ionome profiles of the tested mutants are surely underestimated in the present work, for the following reasons:

a)

The heterozygosity of the *atfh* mutant used for the present analysis, which still possesses one wt AtFH allele.

b)

The time-consuming nature of the preliminary PCR screening work, necessary for the identification of the *atfh* and *atfer4-1/atfh* progeny plants that could be used for each experimental analysis described in the present manuscript. Although hundreds of plants were classified because of that, the availability of an even more plants would have allowed a larger data set analysis with better statistics. Such limitations might be also responsible for lack of clearcut results in PCA analysis.

In past years we tried to circumvent the embryo lethality associated with the complete loss of frataxin in *Arabidopsis* (Vazzola et al., 2007) by transforming *Arabidopsis* plants with inducible

constructs (dexametasone or ethanol induced) for frataxin silencing, by applying artificial microRNA interference technology (amiRNA). However, we could never recover transformed plants stably suppressing frataxin expression, upon treatment with either ethanol or dexamethasone, with any of the different constructs tested and with any of the amiRNA sequences used for that purpose. We could occasionally observe a reduction of AtFH transcript in the treated amiRNA plants but transcript levels increased back to control levels, in successive days of treatment, suggesting that frataxin silencing, when it occurred, was itself short-lived, therefore unsuitable for our research purposes (V. Vazzola, L. Ramirez, I. Murgia, unpublished observations).

It is known that frataxin deficiency is associated with Fe accumulation in mitochondria (Murgia et al., 2009 and references therein), however embryo lethality could not be circumvented by growing heterozygous *A. atfh* mutants under extremely severe Fe deficiency, in alkaline soils up to pH 8.5, as embryo lethality could be indeed still observed in the tiny siliques produced by the plants (data not shown). We also grew *atfh* mutants in another type of Fe-deficiency condition, i.e. on MS medium supplemented with increasing concentrations of the Fe-chelant ferrozine (200–400 μM); again, their siliques contained, when inspected at the microscope with Nomarsky optics, embryos which died early during development (data not shown). Altogether, these results indicate that embryo lethality in *atfh* mutants cannot be bypassed by drastically reducing Fe availability to plants.

Although all the approaches for circumventing the experimental hurdle of embryo lethality failed, the isolation of the *atfer4-1/atfh* mutant described in the present work and its analysis with previously identified single *atfer4-1* (Tarantino et al., 2010a, 2010b) and *atfh* mutants (Vazzola et al., 2007) uncovered potential effects of both ferritin and frataxin in the control of ionome homeostasis; an example of that is molybdenum.

This transition metal, required by nearly all organisms including plants in the form of molybdenum cofactor (Moco), takes part in important metabolic pathways, such as nitrogen assimilation, ABA synthesis, and purine catabolism (Bittner and Mendel, 2010). The existence of a crosstalk between Mo and Fe metabolisms has been recently proposed (for details refer to Bittner, 2014). Indeed, it is known that both Moco synthesis and cytosolic Fe–S assembly depend on mitochondrial-synthesized precursors exported from the organelle by the same transporter, the ATP-binding cassette transporter ATM3 in *Arabidopsis* (Schaedler et al., 2014).

Molybdenum concentration is higher in the *atfer4-1/atfh* mutant than in all the other tested lines, in control soil. Such a difference is not observed any longer when the double mutant is grown in +Fe soil. These results support the hypothesis (Bittner, 2014) that Mo homeostasis is linked to Fe homeostasis.

The observed alterations of Mo profiling in the double mutant with could imply, as a consequence, an alteration in the physiological responses involving Molybdo-enzymes such as Nitrate Reductase (NR), Aldehyde Oxidase (AO) catalyzing the last step of ABA biosynthesis and Xanthine Dehydrogenase XDH (Bittner, 2014). It will be interesting to test such mutants in stress conditions directly involving these enzyme activities, such as for example, during drought stress in order to verify whether the alteration in Mo concentration observed in the double mutants has, as consequence, an altered physiological response to such stress.

Zinc is an essential micronutrient for plants, with multiple roles in various cell compartments; in mitochondria, Zn is part of the prosthetic group of complex IV, of metalloproteases and of proteins involved in translocation (two small TIM proteins) (Nouet et al., 2011). Unlike the transition metal Fe, which is redox active, Zn is instead a structural component of proteins (Nouet et al., 2011). The

observed alterations of Zn profiling in the double mutant might therefore suggest a structural impact of AtFH and AtFer4 gene impairments on plant cells and in particular on plant mitochondria, aimed at adjusting the changes due to altered Fe homeostasis, which is under control of both frataxin and ferritin (Ramirez et al., 2011).

In conclusion, we investigated the single or synergistic contribution of ferritin and frataxin in affecting the leaf ionome, in different conditions of Fe supply. Our results confirm the existence of an Fe nutritional status undetectable through the measurements of Fe concentration only, the relevance of AtFH and AtFer4 genes in influencing the leaf ionome, as well as the existence of an already proposed crosstalk between Fe and Mo (Baxter et al., 2008; Bittner, 2014). The reasons for the observed changes of leaf ionome profiling, associated with altered expression of AtFH and AtFer4 genes, call for further investigation; however, it is known that the alteration of Fe homeostasis in mitochondria can trigger biochemical as well as structural changes (Ramirez et al., 2011; Vigani et al., 2015); it is therefore conceivable that such alterations might, in turn, extensively affect ions' uptake, transport and distribution.

Author contributions

Conceived and designed the experiments: IM. Performed the experiments: IM and GV. Analysed the data: IM, GV. Wrote the paper: IM.

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Appendix A. Supplementary data

The following are the supplementary data related to this article:

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Fig. S5. Principal Component Analysis (PCA) of ionome profile in leaves of four weeks old Col, *atfer4-1, atfh, atfer4-1/atfh* plants grown in control or + Fe soil. Graphs for the three Principal components (PC1 versus 2; PC1 vs 3; PC2 vs 3) accounting for 65% total variance are reported.

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