Plant Soil (2009) 325:25–38
DOI 10.1007/s11104-009-0069-z

REGULAR ARTICLE

Micro-analytical, physiological and molecular aspects of Fe acquisition in leaves of Fe-deficient tomato plants re-supplied with natural Fe-complexes in nutrient solution

Nicola Tomasi · Cecilia Rizzardo · Rossella Monte · Stefano Gottardi · Nahida Jelali · Roberto Terzano · Bart Vekemans · Maria De Nobili · Zeno Varanini · Roberto Pinton · Stefano Cesco

Received: 17 February 2009 / Accepted: 8 June 2009 / Published online: 23 June 2009

Abstract It is well known that in the rhizosphere soluble Fe sources available for plants are mainly a mixture of complexes between the micronutrient and organic ligands such as organic acids and phytosiderophores (PS) released by roots, microbial siderophores as well as fractions of humified organic matter. In the present work, mechanisms of Fe acquisition operating at the leaf level of plants fed with different Fe-complexes were investigated at the micro-analytical, physiological and molecular levels. Fe-deficient tomato plants (*Solanum Lycopersicum* L., cv. 'Marmande') were fed for 24 h with a solution (pH 7.5) containing 1 μM Fe as Fe-PS, Fe-citrate or Fe-WEHS. Thereafter, leaf tissue was used for the visualization of Fe distribution, measurements of Fe content, reduction and uptake, and evaluation of expression of Fe-chelate reductase (*LeFRO1*), Fe-transporter (*LeIRT1*) and Ferritin (*Ferritin2*) genes. Leaf discs isolated from Fe-deficient plants treated for 24 h with Fe-WEHS developed higher rates of translocation, Fe-chelate reduction and ^{59}Fe uptake as compared to plants supplied with Fe-citrate or Fe-PS. Leaves of plants treated with Fe-WEHS also showed higher transcript levels of *LeFRO1*, *LeIRT1* and *Ferritin2* genes with respect to plants fed with the other Fe-sources. Data obtained support the idea that the efficient use of Fe complexed to WEHS-like humic fractions involves, at least in part, also the activation of Fe-acquisition mechanisms operating at the leaf level.

Keywords Iron chlorosis · Natural Fe-sources · *Solanum lycopersicum* L. · Humic substances · ^{59}Fe · Synchrotron μ-XRF analyses

Abbreviations

PS	Phytosiderophores
WEHS	Water-extractable humic fraction
μ-XRF	Micro x-ray fluorescence

Responsible Editor: Jian Feng Ma.

N. Tomasi · C. Rizzardo · R. Monte · S. Gottardi · N. Jelali · M. De Nobili · R. Pinton · S. Cesco (✉)
Dipartimento di Scienze Agrarie e Ambientali,
University of Udine,
Via delle Scienze 208,
33100 Udine, Italy
e-mail: cesco@uniud.it

N. Jelali
CBT de Borj-Cedria,
2050 Tunis, Tunisia

R. Terzano
Dipartimento di Biologia e Chimica Agro-forestale
e Ambientale, University of Bari,
70126 Bari, Italy

B. Vekemans
Department of Analytical Chemistry, Ghent University,
9000 Ghent, Belgium

Z. Varanini
Dipartimento di Scienze, Tecnologie e Mercati della Vite
e del Vino, University of Verona,
37029 San Floriano, Italy

Introduction

Iron deficiency is a yield-limiting factor and a worldwide problem in crop production of many agricultural regions, particularly in calcareous soils (Mengel et al. 2001). Theoretically, total soil-Fe content would be sufficient to meet Fe needs of plants; however, most of the Fe in the soil is present as inorganic forms, predominantly goethite, hematite and ferrihydrite, all poorly available for root uptake under aerobic conditions (Lindsay 1974). Thus, the level of plant-available Fe in the soil solution is determined by a variety of natural ligands (organic acids, siderophores of microbial or plant origin, and components of humified organic matter of the soil) that can mobilize Fe from oxides/hydroxides or from Fe-humates (Lindsay and Schwab 1982). It is well accepted that especially in the rhizosphere a mixture of Fe-complexes is present, and various authors have proved that dicotyledonous plants are able to use them, at least in some cases, as a source of this micronutrient via a reduction-based mechanism (Römheld and Marschner 1986a; Hoerdt et al. 2000; Cesco et al. 2002). Despite these clear evidence, these works were performed using extremely different experimental conditions which render very difficult to comprehend what is the contribution of each Fe source to the Fe acquisition by plants. In the framework of a previous study aimed at evaluating the relative contribution of different natural chelates to Fe-acquisition by plants, it has been demonstrated that Fe complexed to a water extractable humic substances fraction (WEHS) could be accumulated in tomato plants at levels 4–5 times higher than when Fe was supplied as Fe-citrate or Fe-PS (Tomasi et al. 2007). Furthermore, a higher up-regulation of Fe-deficiency related genes (*LeFRO1*, *LeIRT1*, *LeIRT2*) was observed at the root level of plants fed with Fe-WEHS as compared with those supplied with other Fe sources.

At the leaf level, a common consequence of Fe shortage is a low chlorophyll content associated with a limited CO_2 fixation activity (Marschner 1995) which is accompanied by an organic-acid export from the roots to the leaves via xylem (López-Millán et al. 2001). Moreover, it has been demonstrated that plant productivity is highly dependent upon the photosynthetic activity which take place in chloroplasts where N and S assimilation also occurs. These metabolic processes, which require Fe-containing enzymes, leads ultimately to the synthesis of a wide variety of organic compounds (like sugars, amino acids, vitamins), therefore impacting the nutritional quality of edible parts of plants (Briat et al. 2007). For these reasons, the amount of Fe allocated at the leaf level could play an important role to achieve crops of high-nutritional quality.

In the present work, using 34-d-old Fe-deficient tomato plants (*Solanum lycopersicum* L., cv 'Marmande') the contribution to Fe-acquisition of different natural chelates (^{59}Fe complexed to barley-born phytosiderophores, citrate or a water-soluble humic fraction, applied at a final Fe concentration of 1 µM for 24 h) was studied, evaluating the micronutrient fraction allocated at the leaf level. Mechanisms of Fe acquisition operating in the leaves of plants supplied with different Fe-complexes at the end of the treatments were also investigated at the physiological and molecular levels.

Materials and methods

Isolation and purification of Water-Extractable Humic Substances (WEHS)

Water extractable humic substances (WEHS) were obtained as reported by Pinton et al. (1998). Briefly, WEHS were extracted from finely ground sphagnum peat (2.5 g) by adding 50 mL of distilled water and shaking for 15 h at room temperature. Thereafter, the suspension was centrifuged at 8000 RPM for 30 min and the supernatant filtered through a Whatman WCN 0.2 µm membrane filter. The resulting solution was acidified to pH 2 with H_2SO_4 and, in order to purify and concentrate the humified fraction, loaded onto a Amberlite XAD-8 column (Ø 20 mm, height 200 mm; Aiken et al. 1979). Adsorbed humic substances were washed with 100 mL of distilled water according to Aiken et al. (1979) and eluted from the column with 0.1 N NaOH. In order to remove the exchangeable metals, the solution was treated with Amberlite IR-120 (H^+ form) up to pH 1–2, and then adjusted to neutrality with 0.1 N NaOH. The humified organic fraction was then freeze-dried before storage and dissolved in distilled water before use at a concentration of 167 mmol organic C L^{-1}. Molecular size distribution analysis has shown that the fraction contained mostly humic substances of molecular weight lower than 1 kDa (Pinton et al. 1998).

Collection of root exudates and quantitative analysis of epi-hydroxymugineic acid (epi-HMA)

Barley seedlings (*Hordeum vulgare* L., cv 'Europa' provided by V. Römheld, Hohenheim University, Stuttgart, D), germinated for 4 days on filter paper moistened with 1 mM $CaSO_4$, were transferred for a further 14 days to an aerated, Fe-free nutrient solution as described by Zhang et al. (1991). From the 8th day of hydroponic culture, root exudates released by Fe-deficient barley plants were collected by transferring plants to 100 ml of aerated distilled water (pH 6.0) for 4 h during the morning (a period of high PS release). Root exudates containing phytosiderophores (mainly *epi*-HMA; Walter et al. 1995) were filtered through a Whatman WCN 0.2 µm membrane filter and then stored at −80°C.

In order to evaluate the *epi*-HMA content, root exudates containing phytosiderophores (pH 6.0) were passed through a cation-exchange resin column filled with Amberlite IR-120B resin (H^+ -form; Sigma-Aldrich; Ma et al. 1999). After washing with distilled water, the PS retained by the cation-exchange resin were eluted with 2 M NH_4OH and then the eluate was concentrated to dryness in a rotary evaporator (at 40°C; Ma et al. 1999).The residue was re-dissolved in 1 ml water and an aliquot of 100 µl was air-dried, derivatized with phenylisothiocyanate (PITC) and analysed (Howe et al. 1999) by HPLC (LC-1000, Jasco, Tokyo, Japan). The HPLC system was equipped with a C18 column (XTerra RP 18; 150 mm long, 4.6 mm i.d., 3 mm particle size; Waters, Milano, Italy), a Borwin-PDATM 1.50 version (JMBS, Grenoble, France) controller and a PU-1580 pump. The UV absorption spectra of eluate components were obtained using a Jasco model UV-VIS MD-1510 photodiode array detector. Purified *epi*-HMA was used as a standard.

Preparation of natural ^{59}Fe-complexes

Iron-(^{59}Fe)-WEHS complex was prepared as described by Cesco et al. (2000) by mixing WEHS fraction with $^{59}FeCl_3$ in 5 mM Mes-NaOH at pH 6.0; ^{59}Fe-PS and ^{59}Fe-citrate was prepared accordingly to von Wirén et al. (1994) by mixing an aliquot of Fe-free (*epi*-HMA)-containing root exudates collected from Fe-deficient barley plants or citrate (10% excess) with $FeCl_3$. The specific activity of ^{59}Fe in the three Fe-sources was 144 KBq µmol^{-1}Fe.

Plant material, growth conditions and plant tissue analysis

Tomato seedlings (*Solanum Lycopersicum* L., cv. 'Marmande superprecoce' from DOTTO SpA, Italy) were germinated for 6 days on filter paper moistened with 1 mM $CaSO_4$ and then grown for 21 days in a continuously aerated nutrient solution (pH adjusted at 6.0 with 1 M KOH) as reported by Pinton et al. (1999) being exposed to 5 µM Fe (Fe-EDTA); thereafter, the plants were transferred for a further week to a Fe-free nutrient solution (Fe-deficient). The nutrient solution were renewed every three days; before the change, the pH of the old nutrient solutions was recorded using a pH meter. The controlled climatic conditions were the following: day/night photoperiod, 16/8; light intensity, 220 µE m^{-2} s^{-1}; temperature (day/night) 25/20°C; RH 70 to 80%.

SPAD index values of fully expanded young leaves was determined using a portable SPAD-502 meter (Minolta, Osaka, Japan). Fe concentration in leaf and root tissues of tomato plants was determined by ICP-AES, after digestion with concentrated HNO_3; root apoplastic Fe pool was removed before the digestion by 1.2 g L^{-1} sodium dithionite and 1.5 mM 2,2′-bipyridyl in 1 mM $Ca(NO_3)_2$ under N_2 bubbling according to the method described by Bienfait et al. (1985). Reduction of Fe(III)-EDTA by the roots of intact plants was measured as described by Pinton et al. (1999) using the bathophenanthrolinedisulfonate (BPDS) reagent (Chaney et al. 1972). Roots were incubated for 30 min in an aerated solution containing 0.5 mM CaSO4, 250 µM Fe-EDTA, 300 µM BPDS, 10 mM Mes-NaOH (pH 5.5) in the dark at 25°C.

At the end of the growing period (34 days), Fe-deficient tomato plants clearly showed visible symptoms of Fe deficiency (at the leaf level: yellowing of the full expanded apical leaves; at the root level: proliferation of lateral roots, increase in the diameter of the sub-apical zone and amplified root hair formation; Photo in Table 1). The deficiency caused also a marked decrease in root and shoot dry matter accumulation; concomitantly, roots were able to lower

Table 1 Dry matter (mg), iron concentration (ppm) and SPAD index values of 34-d-old Fe-sufficient and Fe-deficient tomato plants. Root Fe(III)-chelate reducing activity, pH of nutrient solutions and photos of root and shoot apparatus of Fe-sufficient and Fe-deficient plants, are also reported

	Fe sufficient plants		Fe deficient plants	
	Root	Shoot	Root	Shoot
Dry matter (mg)	51 ± 5	467 ± 39	39 ± 5	243 ± 43
Fe content (ppm)	777 ± 98	105 ± 13	124 ± 8	51 ± 2
SPAD index value	------	29.9 ± 0.8	------	17.9 ± 0.8
Root Fe^{III}-chelate reductase (μmol g^{-1} root FW h^{-1})	0.37 ± 0.12	------	2.89 ± 0.89	------
pH of nutrient solution	7.4 ± 0.2	------	6.7 ± 0.3	------

Data are means ± SD of three independent experiments

the pH of the nutrient solution and developed an enhanced Fe(III)-EDTA reductase activity (Table 1). These observations are consistent with the induction and operation of a response mechanism to Fe shortage typically ascribable to the Strategy I plants

Iron-(^{59}Fe) uptake from natural Fe-sources by roots of intact plants

As reported by Cesco et al. (2002), roots of two intact Fe-deficient tomato plants (34-d-old) were washed with micronutrient-free nutrient solution for 30 min and then transferred to beakers containing 250 mL of a freshly prepared micronutrient-free nutrient solution; ^{59}Fe-PS, ^{59}Fe-citrate or ^{59}FeWEHS was added in order to give a final Fe concentration of 1 μM. The addition to the nutrient solution of 1 μM Fe as Fe-WEHS brought 5 mg org. C L^{-1} of WEHS. In order to limit photo-chemical reduction phenomena of the micronutrient in the nutrient solution (Zancan et al. 2006) added by the Fe-sources, during the entire experiment, beakers has been covered.

The uptake solution was buffered at pH 7.5 with 10 mM Hepes-NaOH and the uptake period was 24 h. Thereafter, plants were transferred to a freshly prepared ^{59}Fe-free nutrient solution for 10 min in order to remove the excess of ^{59}Fe at the root surface and then harvested. Root apoplastic ^{59}Fe pools were removed by 1.2 g L^{-1} sodium dithionite and 1.5 mM 2,2′-bipyridyl in 1 mM $Ca(NO_3)_2$ under N_2 bubbling according to the method described by Bienfait et al. (1985) (treatment repeated 3 times). Root and shoot tissues were oven-dried at 80°C, weighed, ashed at 550°C, and suspended in 1% (w/v) HCl for ^{59}Fe determination by liquid scintillation counting. The ^{59}Fe uptake rate, measured as nmol ^{59}Fe, is referred to the whole plant (root+shoot) and is presented per g dry weight of roots per 1 or 24 h. The ^{59}Fe translocation rate is presented as nmol ^{59}Fe measured in shoot per g of root dry weight per 24 h. The equivalence in ppm of ^{59}Fe taken up by the plants and determined by liquid scintillation after the treatments with the natural ^{59}Fe-sources, was also calculated in tomato roots and shoot.

In order to calculate the contribute of re-supply treatment with the different ^{59}Fe-sources, in roots and shoot of tomato plants before and after the 24 h treatment with unlabelled natural Fe-sources, the concentrations (ppm) of total Fe, determined by

ICP-AES after digestion of the tissues with concentrated HNO_3, were also determined.

Xylem sap collection

Collection of xylem sap was obtained as reported by López-Millán et al. (2009). Briefly, plants were detopped with a razorblade approximately 5 cm above the roots. Stumps were allowed to bleed for 1 min, then exuded fluid was carefully wiped out with paper tissue and the stem was fitted with plastic tubing. Xylem sap was then allowed to bleed into the plastic tubes for 15 min. After this period, samples were immediately collected, filtered through a Whatman WCN 0.2 µm membrane filter and frozen until analysis by ICP-AES (previous digestion with concentrated HNO_3).

Synchrotron µ-XRF analyses on leaf samples

Full expanded young leaves of intact Fe-deficient tomato plants treated as previously described were collected and prepared as described by Terzano et al. (2008). Briefly, leaf tissues were washed with deionized water and immediately frozen in liquid nitrogen; thereafter, they were freeze-dried under vacuum. From these samples, an area of 2 mm^2, corresponding to the intersection between the primary and a secondary vein, were selected and analyzed by synchrotron 2D-scanning µ-XRF. These analyses were carried out at Beamline L at the Hamburger Synchrotronstrahlungslabor (HASYLAB, Hamburg, Germany) focusing the X-rays to a 20 µm X-ray beam by means of a polycapillary lens (X-ray Optical System, Albany, USA), and using an energy of 15.5 keV. An Al filter was placed in front of the detector in order to improve the signals from higher Z (atomic number) trace elements, lowering down the very intense fluorescent radiation from the low Z elements Ca and K. Leaf samples were placed on a motor x-y-z stage with a movement precision of 1 µm and set at an angle of 45° to the incident beam. Fluorescent radiation was collected with a Vortex-EX (Radiant Detection Technologies) Silicon Drift Detector with 50 mm^2 active area. 2D elemental distribution maps were collected with 20 µm step size and dwell times of 1 sec per point. The XRF spectra, that represent the intensity of the fluorescent X-rays emitted by each element from the whole investigated area, were evaluated using the AXIL software package (Vekemans et al. 1994). From the intensity of the fluorescent X-rays emitted by each element , it can give an idea of the amount of emitting atoms present in this part of the leaf. In order to properly compare the spectra, for each spectrum the intensities of the peaks corresponding to the Kα emissions of the element were divided by the intensity of the Kα emission of Br. This was used as an internal standard, since it is unlikely to be influenced by the different treatments. This ratio was calculated for Mn, Fe, Ni, Cu, and Zn.

Iron as well as trace metal concentrations (ppm) in full expanded apical leaves (whole blade without petioles) of tomato plants treated as previously described, were also determined by ICP-AES, after digestion of the tissues with concentrated HNO_3.

Fe(III) reduction and ^{59}Fe uptake by leaf discs

For these experiments, fully expanded young leaves of intact Fe-deficient tomato plants treated for 24 h with a nutrient solution (buffered at pH 7.5 with 10 mM Hepes-KOH) containing 1 µM Fe as Fe-WEHS, Fe-PS or Fe-citrate, were used to excise samples of 30 leaf discs (Ø: 8 mm). Leaves of Fe-deficient tomato plants before the beginning of the treatments with the natural Fe-sources were used as control.

Ferric reduction was determined according to the methods described by Nikolic and Römheld (1999) by incubating leaf discs, washed twice for 10 min in 5 mL of 0.5 mM $CaSO_4$, 250 mM sorbitol, 10 mM Mes-KOH (pH 6.0) and vacuum infiltrated before the experiment, for 40–60 min in continuous orbital shaker, with a solution (5 mL) containing 10 µM Fe (III)-citrate, 0.5 mM $CaSO_4$, 250 mM sorbitol, 100 µM BPDS, 10 mM Mes-KOH (pH 6.0) in the dark at 25°C; blanks without leaf disks were run under the same conditions. Ferric reduction was determined from the formation of the Fe(II)-BPDS complex at 535 nm against blanks after the leaf discs incubation. Considering the light-dependence of this leaf activity (González-Vallejo et al. 2000), additional experiments was also performed exposing the leaf discs to the light (the same used for the plant growth); in this latter case, in order to subtract the extent of photochemical reduction (Larbi et al. 2001) and the effect of reducing compounds released by disks due to their edge, blanks were performed using leaf disks exposed to the light and maintained in ice temperature during the entire experiment.

Fe(III) uptake was evaluated after incubating leaf discs in 5 mL of a solution (0.5 mM $CaSO_4$, 250 mM sorbitol, 10 mM Mes-KOH at pH 6.0) containing 10 µM Fe as ^{59}Fe-labeled Fe(III)-citrate in the dark for 1 h. Iron(^{59}Fe) radioactivity was measured after removal of apoplastic ^{59}Fe with bipyridyl and sodium dithionite for 15 min (Bienfait et al. 1985). Then, the leaf discs were oven dried, ashed at 550°C, and the residues were dissolved in 1 M HCl to measure ^{59}Fe radioactivity by liquid scintillation counting. The ^{59}Fe uptake, measured as nmol ^{59}Fe, is referred to the total amount of ^{59}Fe in leaf discs per gram fresh weight basis of the tissues.

Uptake of Fe(II) was assayed after incubating leaf discs in 5 mL of a solution (0.5 mM $CaSO_4$, 250 mM sorbitol, 10 mM Mes-KOH at pH 6.0) containing 10 µM Fe as $^{59}Fe(II)SO_4$ prepared according to Zaharieva and Römheld (2000) by mixing the radiochemical tracer ($^{59}FeCl_3$ in 10 mM ascorbate) with 10 mM $FeSO_4$ (in 0.04 M HCl). In order to maintain the micronutrient in the ferrous status during the entire experiment, the uptake solution, with the same composition previously described, was added with ascorbate at a final concentration of 1 mM. The experiment was started by adding $^{59}FeSO_4$ (specific activity of ^{59}Fe was 180 KBq µmol^{-1}Fe) into the uptake solution and lasted 30 min. Radioactivity of ^{59}Fe was measured as previously described.

RNA extraction and cDNA synthesis

Fully expanded young leaves of intact Fe-deficient tomato plants treated as previously described were collected, immediately frozen in liquid nitrogen and conserved until further processing at −80°C.

RNA extractions were performed using TRIzol® reagent (Invitrogen, Carlsbad, USA) following manufacturer's instructions, and contaminant genomic DNA were removed using 10 U of DNase I (GE Healthcare, Munich, Germany). The total-RNA samples were cleaned up using the standard phenol:chloroform protocol (Maniatis et al. 1989). One µg of total RNA (checked for quality and quantity using a spectrophotometer, followed by a migration in an agarose gel) of each sample was retrotranscribed using 1 pmol of Oligo d(T)23VN (New England Biolabs, Beverly, USA) and 10 U M-MulV RNase H^{-} for 1 h at 42°C (Finnzymes, Helsinki, Finland) following the application protocol of the manufacturers.

Gene expression analyses

After RNA digestion with 1 U RNase A (USB, Cleveland, USA) for 1 h at 37°C, gene expression analyses were performed by adding 0.1 µl of the cDNA to FluoCycleTM sybr green (20 µl final volume; Euroclone, Pero, Italy) in a DNA Engine Opticon Real-Time PCR Detection (Biorad, Hercules, USA). Primers used (Tm=58°C) were the following: as housekeeping gene: *EF1* (X14449) tggatatgctc cagtgcttg and ttccttacctgaacgcctgt; *IRT1* (AF136579) tcactaggtgcgtcaagcaa and gtaggatgcaaccaccaagg; *FRO1* (AY224079) atccaataaaggcggtgttg and tgcat cagtcccactctgtc, and *Ferritin2* (BE431630) gttgctctcaagggacttgc and ccaccacgcttgttctgata. Each Real-Time RT-PCR was performed 3 times on 2 independent experiments; analyses of real-time result were performed using Opticon Monitor 2 software (Biorad, Hercules, USA) and R (version 2.7.0; http://www.r-project.org/) with the qPCR package (version 1.1–4; http://www.dr-spiess.de/qpcR.html). Efficiencies of amplification were calculated following the authors' indications (Ritz and Spiess 2008): PCR efficiencies were 80.25%, 76.25%, 77.70% and 82.35% for *EF1*, *IRT1, FRO1* and *Ferritin2* genes, respectively.

Statistical analysis

Computation of the graphical representation and statistical validation (Student's t-test; $p<0.05$) were performed using SigmaPlot 11.0 (Systat software, Point Richmond, USA). Gene expression data were illustrated considering the differences in the PCR efficiency of amplification and using the gene expression levels in leaves of untreated Fe-deficient plants (control) as reference.

Results

In order to study the capability of tomato plants to utilize natural Fe-sources, ^{59}Fe-uptake experiments were performed incubating roots of intact Fe-deficient plants in a nutrient solution (pH 7.5) for 24 h in the presence of ^{59}Fe (final concentration 1 μM) complexed to barley phytosiderophores (PS), citrate or a water-soluble humic fraction (WEHS). Table 2 shows that plants were able to absorb ^{59}Fe from the three sources; however, whole plant ^{59}Fe accumulation was 3.5–4-fold higher when supplying Fe as Fe-WEHS than as Fe-PS or Fe-citrate. At the leaf level, when plants were fed with ^{59}Fe-WEHS, a higher amount of the micronutrient (4.5–7-fold higher) was accumulated than when using the other Fe-sources. Hence, translocation of absorbed ^{59}Fe was enhanced by Fe-WEHS treatment (35% vs 26% or 21%). In Table 3 (A) the μg of ^{59}Fe per g DW taken up by plants fed with the different natural ^{59}Fe-sources are shown; results confirm the higher plant-use efficiency and translocation of Fe provided as Fe-WEHS than when supplied as Fe-PS or Fe-citrate. The same pattern was also observed when, in additional experiments, Fe-deficient tomato plants were re-supplied with the unlabelled three Fe-sources and the Fe-contents in the roots and shoot were determined by ICP-AES, after digestion of the tissues (Table 3, B). The contribution of ^{59}Fe re-supplied treatments to the total Fe-concentration in the plant tissues were calculated on the basis of data reported in Table 3 (A and B). Results show that this parameter ranged from 14 to 23% or from 2 to 12% at the root or shoot level, respectively, the highest being when plants were fed with ^{59}Fe-WEHS. This is particularly evident at the leaf level (about 4–6 fold higher than those calculated for plants supplied with ^{59}Fe-PS or ^{59}Fe-citrate).

To confirm the change in translocation rates, ICP-AES analyses were also performed on xylem sap samples (Table 4). Results show an increase of Fe concentration after the Fe re-supply treatments, being highest in Fe-deficient plants fed with Fe-WEHS (about 4 fold increase). Higher concentrations of Mn, were also detected in Fe-WEHS treated plants as compared to untreated plants.

In order to get information on the relative distribution of Fe and other micro-elements within the leaf tissues, μ-XRF image maps were acquired using leaf samples over 2 mm^2 areas. In Fig. 1 the μ-XRF elemental maps collected for a representative Fe-deficient control plant sample are reported; a spectrum corresponding to the sum of all the spectra obtained from each spot in the investigated area is also reported. Iron distribution, as well as the distribution of the other elements (K, Ca, Cu, Ni, Zn, Br, Mn), was identical both for Fe-deficient control and Fe re-supplied plants (not shown). Iron was localized in the main veins; a similar behaviour was observed for K, Zn and Br. Iron localization in the main veins with low concentrations in the interveinal mesophyll areas was also observed by Jimenéz et al. (2009) in leaves of Fe-sufficient and Fe-deficient *Prunus*, by using synchrotron μ-XRF. However, compared to the data presented in the present paper, they used a lower resolution (100 vs 20 μm) with a consequent limitation on the level of detail achieved in elemental distribution maps. Calcium was localized in specific spots throughout all the leaf, corresponding to the trichomes. Copper and Ni appeared to be present mainly in the primary and secondary veins; Mn was almost homogeneously distributed over the leaf area. In Table 5 (A) are reported the ratios calculated for Mn, Fe, Ni, Cu, and Zn, in all the leaf samples, using the fluorescent X-rays intensity emitted by each element and, as reference, the Kα emission of Br. The amount of Fe

Table 2 Uptake and translocation of ^{59}Fe by Fe-deficient tomato plants treated at the root level for 24 h (pH 7.5) with ^{59}Fe-PS, ^{59}Fe-citrate or ^{59}Fe-WEHS at a final Fe concentration of 1 μM

	^{59}Fe uptake (whole plant)	Root to shoot ^{59}Fe translocation
	nmol ^{59}Fe g^{-1}root DW in 24 h	
^{59}Fe-source		
^{59}Fe-PS	496±37 B	131±12 B (26)
^{59}Fe-Citrate	422±29 C	88±27 C (21)
^{59}Fe-WEHS	1703±93 A	596±63 A (35)

Values in parenthesis represent percentage of ^{59}Fe translocation to shoots. Data are means ± SD of three independent experiments; capital letters refer to statistically significant differences within each column (t-test, $P<0.05$)

Table 3 Iron-(^{59}Fe) acquired from ^{59}Fe-sources (**A**) (determined by liquid scintillation and expressed in ppm) and total Fe concentration (**B**) (determined by ICP-AES) in roots and shoot of Fe-deficient tomato plants treated for 24 h (pH 7.5) with Fe-PS, Fe-citrate or Fe-WEHS at a final Fe concentration of 1 μM

	A		B	
	^{59}Fe acquired (μg g^{-1} DW) from ^{59}Fe-sources		Total Fe concentration (μg g^{-1} DW)	
	determined by liquid scintillation		determined by ICP-AES	
Fe-source	root	shoot	root	shoot
+Fe-PS	21.3±2.6 B [14.8%]	1.6±0.1 B [3.0%]	143.9±8.6 B	53.2±1.9 B
+Fe-Citrate	19.7±1.9 B [14.1%]	0.9±0.2 C [1.7%]	139.2±11.3 BC	52.7±2.3 B
+Fe-WEHS	40.4±8.8 A [23.4%]	7.2±1.2 A [12.2%]	172.3±13.4 A	59.1±2.9 A

Values in square brackets indicate contribute of resupply treatment of each Fe-sources. Data are means ± SD of three independent experiments; capital letters refer to statistically significant differences within each column (t-test, $P<0.05$)

in leaves of Fe-WEHS fed plants was estimated to be about 5 times higher than that of Fe-deficient control plants; only a slight increase in Fe amount was recorded in leaves of plants treated with Fe-PS or Fe-citrate. Higher amounts of Mn, Ni, Cu and Zn were also detected in Fe-WEHS treated plants as compared to the other Fe treatments. In Table 5 (B) the trace-element concentrations (μg g^{-1}DW) determined by ICP-AES in full expanded apical leaves (whole blade without petioles) are reported. Although the two analytical approaches analyzed diverse areas of the leaf blade, results obtained by ICP-AES also showed that the concentration levels of trace metal in leaves of plants fed with Fe-WEHS were significantly higher than those recorded in Fe-deficient plants treated with Fe-PS or Fe-citrate, confirming what was observed by synchrotron analyses. The Fe re-supply treatments caused also a recovery of the SPAD index values of the apical leaves, the highest being when Fe-deficient plants were supplied with Fe-WEHS (Table 5, B).

In order to evaluate the functionality of Fe-acquisition mechanisms working at the leaf level of plants supplied with different Fe-complexes, Fe(III)-citrate reduction, ^{59}Fe(III)-citrate uptake and $^{59}FeSO_4$ uptake were measured using leaf discs excised from fully expanded young leaves of intact Fe-deficient tomato plants treated in the nutrient solution for 24 h with 1 μM Fe as Fe-WEHS, Fe-PS or Fe-citrate and adopting assay conditions reported by Nikolic and Römheld (1999). Figure 2a shows that supply of Fe caused a higher capacity of leaf tissues to reduce exogenous Fe(III)-citrate; however, reduction activity was significantly higher than that measured in control Fe-deficient plants only in leaves of plants fed with Fe-WEHS. For the dependence on the light of this leaf activity (González-Vallejo et al. 2000), Fe(III)-

Table 4 Concentrations of cationic nutrients (determined by ICP-AES) in xylem sap samples of Fe-deficient tomato plants treated for 24 h (pH 7.5) with Fe-PS, Fe-citrate or Fe-WEHS at a final Fe concentration of 1 μM

Nutrient	Control Fe-deficient	Fe-PS	Fe-Citrate	Fe-WEHS
	μg mL^{-1}			
Mn	0.031±0.008 B (100)	0.029±0.005 B (93)	0.037±0.014 AB (119)	0.057±0.010 A (184)
Fe	0.449±0.088 C (100)	0.655±0.024 B (146)	0.578±0.190 BC (129)	1.999±0.362 A (445)
Zn	0.591±0.174 AB (100)	0.419±0.120 B (71)	0.624±0.272 AB (106)	0.647±0.113 A (109)
K	1045±60 A (100)	760±106 B (73)	879±84 AB (84)	979±49 A (94)
Mg	217±19 A (100)	171±13 C (79)	191±5 B (88)	205±11 AB (94)

Values in parenthesis represent percentage of–Fe control leaf. Data are means ± SD of two independent experiments; capital letters refer to statistically significant differences within each line (t-test, $P<0.05$)

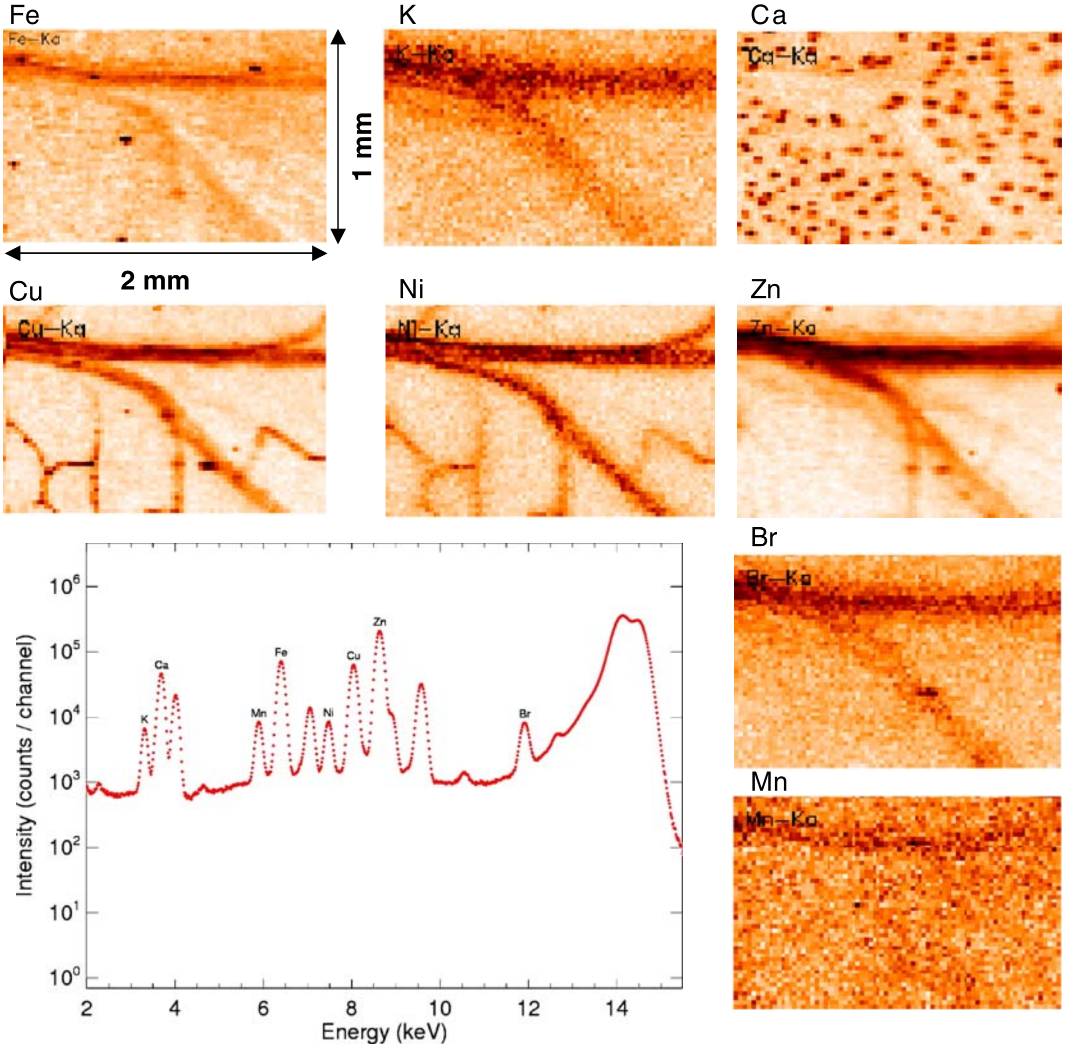

Fig. 1 Fe, K, Ca, Cu, Ni, Zn, Br, and Mn distributions on a 2 mm^2 area of a leaf imaged by 2-D scanning µ-XRF. The µ-XRF elemental maps is referred to a representative Fe-deficient control plant sample. Darker pixels correspond to areas with a relatively higher element concentration. The sum-spectrum corresponding to the same area is also reported. The Kα emission peak for each element is indicated

citrate reduction was also evaluated exposing the leaf discs to light. Results reported in Fig. 2b confirm those obtained in the dark experiments. In Fig. 3 the levels of Fe uptake evaluated incubating in darkness leaf discs in a solution containing 10 µM ^{59}Fe(III)-citrate or $^{59}FeSO_4$, are reported. Results show that only the treatment of tomato plants with Fe-WEHS determined a significant increase in Fe uptake from ^{59}Fe(III)-citrate (Fig. 3a); on the other hand, no effect due to the treatment with the different natural Fe sources was recorded in the uptake rates of Fe(II) from $^{59}FeSO_4$ (Fig. 3b).

To evaluate the involvement of a transcriptional regulation of Fe-uptake mechanisms at the leaf level, mRNA abundance of *LeFRO1* (coding for an isoform of the PM Fe(III)-chelate reductase) and *LeIRT1* (coding for Fe^{2+} transporter) were analyzed in leaves of intact Fe-deficient tomato plants treated for 24 h with Fe-WEHS, Fe-PS or Fe-citrate in nutrient solution. Results reported in Fig. 4 show that the relative expression levels of the two genes, evaluated by real-time RT-PCR, were influenced by the treatments. In fact, in leaf cells of Fe-deficient plants fed with Fe-WEHS a significant increase of *LeIRT1* and

Table 5 **A**: Trace element relative peak intensity (Br used as reference peak) calculated for samples (area of 2 mm^2) isolated from full expanded apical leaves. **B**: SPAD index values and trace element concentration (ppm) of full expanded apical leaves (whole blade without petioles) determined by ICP-AES after its digestion

Trace element	Control Fe-deficient	Fe-PS	Fe-Citrate	Fe-WEHS
	A			
			I/I[Br-Kα]	
Mn	0.4±0.1 B (100)	0.5±0.2 B (125)	0.4±0.2 B (100)	1.6±0.4 A (400)
Fe	3.5±0.4 C (100)	3.9±0.4 BC (111)	4.4±0.3 B (126)	16±3 A (457)
Ni	0.5±0.3 B (100)	0.6±0.2 B (120)	0.4±0.2 B (80)	1.9±0.6 A (380)
Cu	2.7±0.4 C (100)	2.6±0.5 C (96)	4.0±0.5 B (148)	14±3 A (518)
Zn	15±3 C (100)	17±4 B (113)	13±3 C (87)	66±5 A (440)
	B			
			$\mu g\ g^{-1}$ DW	
Mn	16.3±0.4 B (100)	17.6±0.3 AB (108)	17.1±0.5 B (105)	18.8±1.0 A (115)
Fe	59.1±0.9 C (100)	63.5±2.1 B (107)	65.1±4.1 B (110)	79.2±3.8 A (134)
Ni	2.9±0.4 B (100)	3.2±0.3 B (110)	3.0±0.3 B (103)	4.7±0.4 A (162)
Cu	18.2±0.2 B (100)	17.7±0.7 B (97)	21.7±2.0 A (120)	25.1±1.8 A (138)
Zn	44.9±6.1 B (100)	49.7±7.3 AB (111)	37.1±13.2 B (83)	65.5±11.3 A (146)
SPAD index	17.9±0.8 C	19.8±0.3 AB	19.2±0.5 BC	21.0±1.2 A

Values in parenthesis represent percentage of–Fe control leaf. Data are means ± SD of three independent analyses; capital letters refer to statistically significant differences within each line (t-test, $P<0.05$)

LeFRO1 gene expression levels occurred. A slight but significant increase in transcript abundance of the two genes was also recorded in plants fed with Fe-PS and Fe-citrate. Evaluation of expression level of *Ferritin2* gene was also performed showing that higher Fe accumulation in leaves of Fe-WEHS treated plants was accompanied by an up-regulation of the gene (Fig. 5).

Discussion

The ability of plants to take up Fe from the soil depends on their capacity to utilize natural soluble sources and/or to mobilize sparingly soluble Fe forms (Römheld and Marschner 1986b). This task is accomplished by releasing Fe-chelating substances (i.e. PS, citrate) able to form Fe(III)-complexes which in turn act as substrates for root-uptake mechanisms (Römheld and Marschner 1986a). Furthermore, fractions of humified organic matter may be present in the soil solution, providing an additional soluble Fe source directly utilizable by plants (Chen 1996; Pandeya et al. 1998; Varanini and Pinton 2006). While mechanisms adopted by plants to take up Fe from complexes like Fe-PS and Fe-WEHS have been well described (Römheld and Marschner 1986a; Cesco et al. 2002, 2006), poor evidence is available on the use efficiency of these different natural Fe sources in a context more similar to what is occurring in the rhizosphere.

In the present work we compared three natural Fe complexes, namely Fe-PS, Fe-citrate and Fe-WEHS, with respect to their capacity to provide Fe to Fe-deficient tomato plants; particularly, accumulation at the leaf level was evaluated after supplying for 24 h each Fe source to the nutrient solution. Furthermore, the functioning of Fe-uptake mechanisms (Fe(III) reduction and Fe(II) uptake) was assayed using leaf discs isolated from control and treated plants and correlated to gene expression analyses. Results show that Fe-deficient tomato plants are able to utilize the three Fe sources; however, when plants were put in contact with Fe-WEHS, a higher Fe accumulation in the whole plant was recorded, with root-shoot translocation showing a more than proportional increase (Tables 2, 3 and 4). These data confirm previous observation that Fe(III)-WEHS could act as

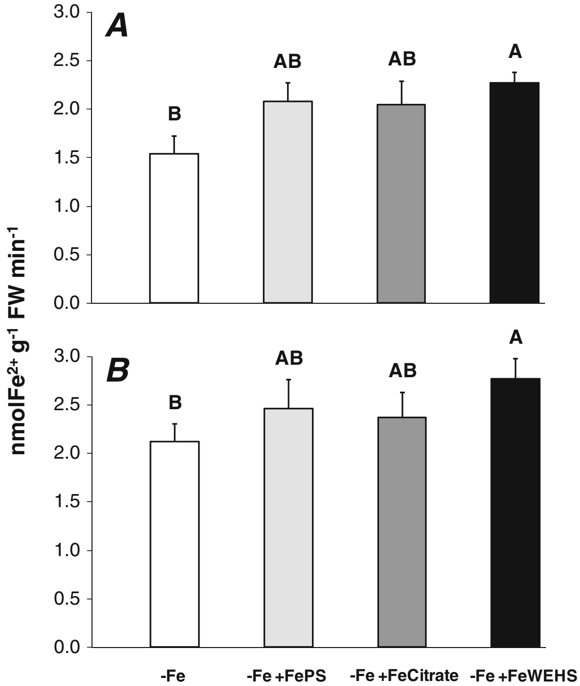

Fig. 2 Fe(III)-reduction activity of leaf discs isolated from full expanded young leaves of intact 34-d-old Fe-deficient tomato plants supplied in nutrient solution (pH 7.5) for 24 h with Fe-PS, Fe-citrate or Fe-WEHS at a final Fe concentration of 1 µM; as control, leaf discs of Fe-deficient plants not treated with any Fe sources were utilized. Reduction experiment was carried out in dark (*Plate A*) or in light (*Plate B*) in 0.5 mM $CaSO_4$, 250 mM sorbitol, 300 µM BPDS, 10 mM Mes-KOH (pH 6.0) assay solution supplied with 10 µM Fe(III)-citrate (Fe/citrate ratio 1/1.1). *Bars* represent means ± SD of three independent experiments; *capital letters* refer to statistically significant differences between treatments (t-test, $P<0.05$)

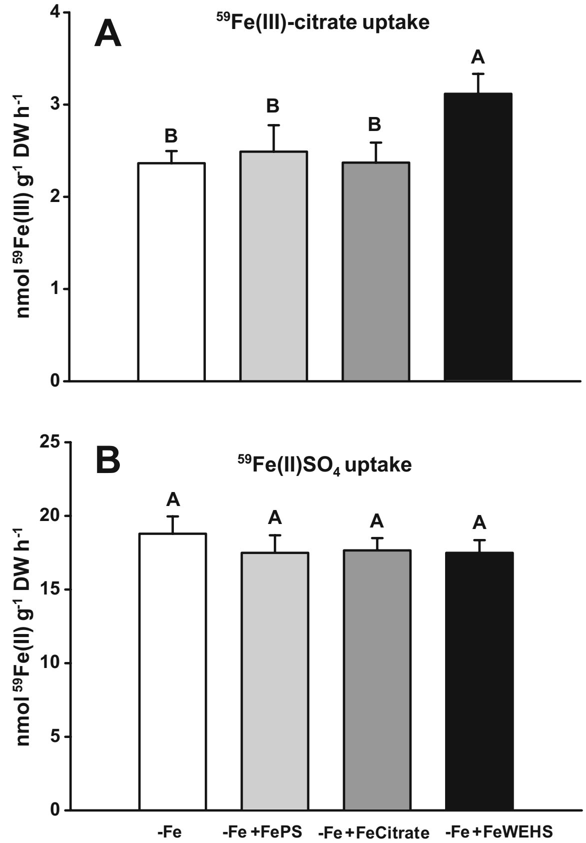

Fig. 3 Uptake of Fe(III) (**a**) and Fe(II) (**b**) by leaf discs isolated from full expanded young leaves of intact 34-d-old Fe-deficient tomato plants supplied in nutrient solution (pH 7.5) for 24 h with Fe-PS, Fe-citrate or Fe-WEHS at a final Fe concentration of 1 µM; as control, leaf discs of Fe-deficient plants untreated with any Fe sources, were utilized. Experiments were carried out in dark and in 0.5 mM $CaSO_4$, 250 mM sorbitol, 10 mM Mes-KOH (pH 6.0) assay solution; Fe(III) and Fe(II) uptake were evaluated supplying to the assay medium 10 µM Fe as ^{59}Fe(III)-citrate or $^{59}FeSO_4$, respectively. *Bars* represent means ± SD of three independent experiments; *capital letters* refer to statistically significant differences between treatments (t-test, $P<0.05$)

a suitable substrate for the PM Fe(III)-chelate reductase (Pinton et al. 1999, Cesco et al. 2002). The different use efficiency here recorded could be due to different stabilities of the three Fe complexes at pH 7.5; the similar utilization by roots of Fe-citrate and Fe-PS, whose stability constants are different for several orders of magnitude (10^8 [Jones 1998] vs 10^{18} [Sugiura et al. 1981], respectively) indicates that this sole parameter is not sufficient to explain the phenomenon. Furthermore, the higher affinity of PS for Fe than WEHS (Cesco et al. 2000) is not associated to the higher use efficiency of Fe-PS complexes by the roots. Localized pH decrease of root-external medium, one of the mechanisms through which dicots, including tomato, respond to the Fe shortage (Table 1), could surely contribute to create at the root surface conditions more favorable for Fe acquisition from the different sources.

Leaf analysis by µ-XRF (Fig. 1) showed that Fe distribution within this tissue (2 mm^2 interveinal area), was not influenced by the nature of the source (data not shown); on the other hand, this approach allowed to confirm that a higher amount of Fe was allocated to the leaves in Fe-WEHS treated plants (Table 4, A). This behaviour, also supported by ICP-AES analyses of the whole leaf blade (Table 4 B), could be also observed for Ni, Cu, Zn and Mn, indicating that a fast recovery of nutritional and growth limitation was occurring in plants treated with

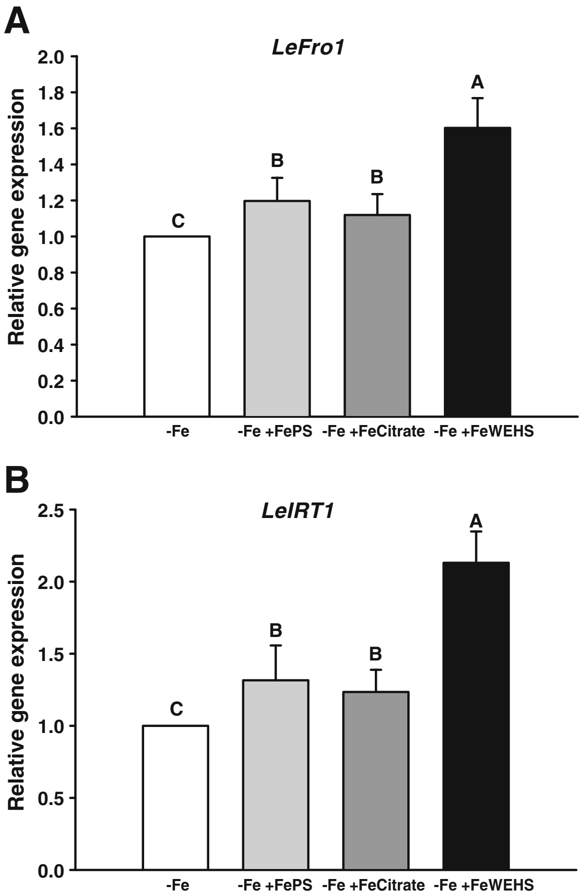

Fig. 4 Real-time RT-PCR analyses of *LeIRT1* (**a**) and *LeFRO1* (**b**) genes expression in full expanded young leaves of intact 34-d-old Fe-deficient tomato plants supplied in nutrient solution (pH 7.5) for 24 h with Fe-PS, Fe-citrate or Fe-WEHS at a final Fe concentration of 1 µM; as control, leaf discs of Fe-deficient plants not treated with any Fe sources, were utilized. Gene mRNA levels were normalized with respect to the internal control *EF1*; relative changes in gene expression were calculated on the basis of their expression levels in Fe-deficient plants. *Bars* represent means ± SD of transcript levels on 2 independent experiments with 3 replicates; *capital letters* refer to statistically significant differences between treatments (t-test, $P<0.05$)

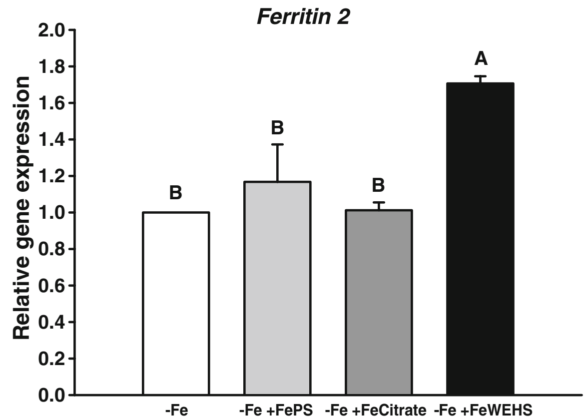

Fig. 5 Real-time RT-PCR analyses of *Ferritin2* gene expression in full expanded young leaves of plants treated as reported in Fig. 4. Gene mRNA levels were normalized with respect to the internal control *EF1*; relative changes in gene expression were calculated on the basis of their expression levels in Fe-deficient plants. *Bars* represent means ± SD of transcript levels on 2 independent experiments with 3 replicates; *capital letters* refer to statistically significant differences between treatments (t-test, $P<0.05$)

Fe-humic complex (Pinton et al. 1999; Nikolic et al. 2007). A higher allocation at the leaf level in Fe-WEHS treated plants was further supported by both the higher Fe concentration in the xylem sap (Table 4) and the higher expression of the gene (*Ferritin2*) coding for a ferritin isoform (Fig. 5).

Leaf discs of Fe-WEHS treated plants showed also the highest rates of Fe(III)-chelate reductase activity and ^{59}Fe uptake (Figs. 2 and 3a). This behavior is consistent with the higher transcript abundance of a reductase gene (*LeFRO1*) observed in the leaf tissue (Fig. 4a) and the higher expression of Fe-transporter gene (*LeIRT1*) in leaves of Fe-WEHS fed plants (Fig. 4b), although direct uptake of Fe^{2+} (i.e. independent from Fe(III) reduction) did not show any significant difference among treatments (Fig. 3b). With respect to this latter data is worth to note that post-transcriptional regulation has been reported both for the Fe(III) reductase and the Fe^{2+} transporter (Connolly et al. 2002, 2003). Interestingly, an up-regulation of Fe transporters (*LeIRT1* and *LeIRT2*) and reductase (*LeFRO1*) genes has been also reported at the root level following Fe-WEHS addition to the nutrient solution (Tomasi et al. 2007). In addition, different regulation in the activity levels of the two components of Fe acquisition (Fe(III)-chelate reductase and subsequent uptake of Fe^{2+} ions via trans-membrane transporter) in Fe-deficient plants upon variable N availability, has been documented at the root level by Nikolic et al. (2007). Interestingly, also in barley (Strategy II plant species) the low release of phytosiderophores by roots of Fe-sufficient plants (first component of Fe acquisition) is not strictly associated to the low capability of roots to take up exogenous Fe-PS complexes (the second component involving a transmembrane transporter) (Cesco et al. 2002), unless plants were exposed to S starvation, which limited the uptake of exogenous Fe-PS complexes from the external medium (Astolfi et al. 2006).

In conclusion, data of the present work show that Fe use efficiency is also dependent on the Fe source available for plant uptake. In addition, we could provide evidence that a better use of Fe complexed to WEHS-like fractions as compared to other natural complexes involves, at least in part, activation of Fe-uptake mechanisms operating at the leaf level. This result might be to take in consideration for Fe (and possibly other micronutrients) bio-fortification (Wu et al. 2008).

Acknowledgements Research was supported by grant from Italian *M.U.R.S.T.*. We thank: Dr. Adamo Domenico Rombolà (University of Bologna) for performing PS analysis, Dr. J.F. Ma (Kagawa University) for providing purified *epi*-HMA, Prof Volker Römheld (Hohenheim University) for providing barley seeds cv 'Europa', Dr. Filip Pošćić (University of Udine) for performing ICP-AES analyses. Synchrotron experiments at HASYLAB were financially supported by the European Community-Research Infrastructure Action under the FP6 "Structuring the European Research Area" Program (Integrating Activity on Synchrotron and Free Electron Laser Science; Contract RII3-CT-2004-506008). This part of the research was also performed as part of the "Interuniversity Attraction Poles" programme financed by the Belgian government.

References

Aiken GR, Thurman EM, Malcolm R (1979) Comparison of XAD macroporous resin for the concentration of fulvic acid from aqueous solution. Anal Chem 51:1799–1803

Astolfi S, Cesco S, Zuchi G et al (2006) Sulphur starvation reduces phytosiderophores release by Fe-deficient barley plants. Soil Sci Plant Nutr 52:43–48

Bienfait HF, Van den Briel W, Mesland-Mul NT (1985) Free space iron pools in roots: generation and mobilization. Plant Physiol 78:596–600

Briat JF, Curie C, Gaymard F (2007) Iron utilization and metabolism in plants. Curr Op Plant Biol 10:276–282

Chaney RL, Brown JC, Tiffin LO (1972) Obligatory reduction of ferric chelates in iron uptake by soybeans. Plant Physiol 50:208–213

Connolly EL, Fett JP, Guerinot ML (2002) Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. Plant Cell 14:1347–1357

Connolly EL, Campbell NH, Grotz N et al (2003) Over-expression of the FRO2 ferric chelate reductase confers tolerance to growth on low iron uncovers posttranscriptional control. Plant Physiol 133:1102–1110

Cesco S, Römheld V, Varanini Z et al (2000) Solubilization of iron by water-extractable humic-substances. J Plant Nutr Soil Sci 163:285–290

Cesco S, Nikolic M, Römheld V et al (2002) Uptake of ^{59}Fe from soluble ^{59}Fe-humate complexes by cucumber and barley plants. Plant Soil 241:121–128

Cesco S, Rombolà AD, Tagliavini M et al (2006) Phytosiderophores released by graminaceous species promote ^{59}Fe uptake in citrus. Plant Soil 287:223–233

Chen Y (1996) Organic matter reactions involving micronutrients in soils and their effect on plants. In: Piccolo A (ed) Humic substances in terrestrial ecosystems. Elsevier Science B.V, Amsterdam, pp 507–529

González-Vallejo EB, Morales F, Cistué L, Abadía A, Abadía J (2000) Iron deficiency decreases the Fe(III)-chelate reducing activity of leaf protoplasts. Plant Physiol 122:337–344

Hoerdt W, Römheld V, Winkelmann G (2000) Fusarinines and dimerum acid, mono- and dihydroxamate siderophores from Penicillum chrysogenum, improve iron utilization by strategy I and strategy II plants. Biometals 13:37

Howe JA, Choi YH, Loeppert RH et al (1999) Column chromatography and verification of phytosiderophores by phenylisothiocyanate derivatization and UV detection. J Chromatog. 841:155–164

Jimenéz S, Morales F, Abadia A, Abadia J, Moreno MA, Gocorcena Y (2009) Elemental 2-D mapping and changes in leaf iron and chlorophyll in response to iron re-supply in iron-deficient GF 677 peach-almond hybrid. Plant Soil 315:93–106

Jones DL (1998) Organic acids in the rhizosphere–A critical review. Plant Soil 205:25–44

Larbi A, Morales F, López-Millán A et al (2001) Technical advance: Reduction of Fe(III)-chelates by mesophyll leaf disks of sugar beet. Multi-component origin and effects of Fe deficiency. Plant Cell Physiol 42:94–105

Lindsay WL (1974) Role of chelation in the micronutrient availability. In: Carson EW (ed) The plant root and its environment. University Press of Virginia, Charlottesville, pp 507–524

Lindsay WL, Schwab AP (1982) The chemistry of iron in soils and its availability to plants. J Plant Nutr 5:821–840

López-Millán AF, Morales F, Abadia A et al (2001) Changes induced by Fe deficiency and Fe resupply in the organic acid metabolism of sugar beet (*Beta vulgaris*) leaves. Physiol Plant 112:31–38

López-Millán AF, Morales F, Gogorcena Y et al (2009) Metabolic responses in iron deficient tomato plants. J Plant Physiol 166:375–384

Maniatis T, Sambrook J, Fritsch EF (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York

Ma JF, Taketa S, Chang Y et al (1999) Genes controlling hydroxylations of phytosiderophores are located on different chromosomes in barley (*Hordeum vulgare* L.). Planta 207:590–597

Marschner H (1995) Mineral nutrition of higher plants, 2nd edn. Academic, London

Mengel K, Kirkby E, Kosegarten H et al (2001) Iron. In Mineral Nutrition. Kluwer, Dordrecht, pp 553–571

Nikolic M, Römheld V (1999) Mechanism of Fe uptake by the leaf symplast: is Fe inactivation in leaf a cause of Fe deficiency chlorosis? Plant Soil 215:229–237

Nikolic M, Cesco S, Varanini Z et al (2007) Short-term interactions between nitrate and iron nutrition in cucumber. Funct Plant Biol 34:402–408

Pandeya SB, Singh AC, Dhar P (1998) Influence of fulvic acid on transport of iron in soils and uptake by paddy seedlings. Plant Soil 198:117

Pinton R, Cesco S, De Nobili M et al (1998) Water and pyrophosphate-extractable humic substances as a source of iron for Fe-deficient cucumber plants. Biol Fert Soil 26:23–27

Pinton R, Cesco S, Santi S et al (1999) Water-extractable humic substances enhance iron deficiency responses by Fe-deficient cucumber plants. Plant Soil 210:145–157

Ritz C, Spiess AN (2008) qpcR: an R package for sigmoidal model selection in quantitative real-time polymerase chain reaction analysis. Bioinform 24:1549–1551

Römheld V, Marschner H (1986a) Evidence for a specific uptake system for iron phytosiderophores in roots of grasses. Plant Physiol 80:175–180

Römheld V, Marschner H (1986b) Mobilization of iron in the rizosphere of different plant species. In: Tinker B, Laüchli A (eds) Advances in plant nutrition. Praeger Scientific, New York, pp 155–204

Sugiura Y, Tanaka H, Miro Y et al (1981) Structure, properties and transport mechanism of iron(III) complex of mugineic acid, a possible phytosiderophore. J Am Chem Soc 103:6979–6982

Tomasi N, De Nobili M, Varanini Z et al (2007) Use efficiency of natural iron complexes by tomato plants. International Symposium “Rhizosphere 2”, Montpellier, France, 145, P-1075

Terzano R, Al Chami Z, Vekemans B et al (2008) Zinc distribution and speciation within rocket plants (*Eruca vesicaria* L. Cavalieri) grown on a polluted soil amended with compost as determined by XRF microtomography and micro-XANES. J Agric Food Chem 56:3222–3231

Varanini Z, Pinton R (2006) Plant-Soil Relationship: Role of Humic Substances in Iron Nutrition. In: Barton LL, Abadía J (eds) Iron nutrition in plants and rhizospheric microorganisms. Springer Verlag, Heidelberg, pp 153–168

Vekemans B, Janssens K, Vincze L et al (1994) Analysis of X-ray spectra by iterative least squares (AXIL)–New developments. X-Ray Spectrom 23:278–285

von Wirén N, Mori S, Marschner H et al (1994) Iron inefficiency in maize mutant ys1 (*Zea mays* L. cv Yellow-Stripe) is caused by a defect in uptake of iron phytosiderophores. Plant Physiol 106:71–77

Walter A, Pich A, Scholz G et al (1995) Diurnal variation in release of phytosiderophores and in concentration of phytosiderophores and nicotianamine in roots and shoots of barley. J Plant Physiol 147:191–196

Wu L, Zhang J, Miller DD et al (2008) Iron enrichment in rice (*Oriza sativa* L.) grain using a foliar high-efficiency iron containing fertilizer in a crop-soil system. Abstract of 14th International Symposium on Iron Nutrition and Interaction in Plants, Beijing, China, 141

Zaharieva T, Römheld V (2000) Specific Fe^{2+} uptake system in strategy I plants inducible under Fe deficiency. J Plant Nutr 23:1733–1744

Zancan S, Cesco S, Ghisi R (2006) Effect of UV-B radiation on iron content and distribution in maize plants. Environm Exp Bot 55:266–272

Zhang FS, Römheld V, Marschner H (1991) Role of the root apoplasm for iron acquisition by wheat plants. Plant Physiol 97:1302–1305