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Review

Towards a knowledge-based correction of iron chlorosis

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ABSTRACT

Iron (Fe) deficiency-induced chlorosis is a major nutritional disorder in crops growing in calcareous soils. Iron deficiency in fruit tree crops causes chlorosis, decreases in vegetative growth and marked fruit yield and quality losses. Therefore, Fe fertilizers, either applied to the soil or delivered to the foliage, are used every year to control Fe deficiency in these crops. On the other hand, a substantial body of knowledge is available on the fundamentals of Fe uptake, long and short distance Fe transport and subcellular Fe allocation in plants. Most of this basic knowledge, however, applies only to Fe deficiency, with studies involving Fe fertilization (i.e., with Fe-deficient plants resupplied with Fe) being still scarce. This paper reviews recent developments in Fe-fertilizer research and the state-of-the-art of the knowledge on Fe acquisition, transport and utilization in plants. Also, the effects of Fe-fertilization on the plant responses to Fe deficiency are reviewed. Agronomical Fe-fertilization practices should benefit from the basic knowledge on plant Fe homeostasis already available; this should be considered as a long-term goal that can optimize fertilizer inputs, reduce grower's costs and minimize the environmental impact of fertilization.

1. Introduction

Iron (Fe) deficiency chlorosis is a major nutritional disorder in crops growing on calcareous soils. This deficiency is particularly important in fruit tree species, causing decreases in tree vegetative growth, marked fruit yield and quality losses and a decrease in the life span of orchards (for reviews see [1,2]). Therefore, Fe fertilizers, either applied to the soil or delivered to the foliage, are provided to these crops every year to control Fe deficiency, and the use of Fe-fertilization is increasing. The amounts of Fe needed depend on the crop, and in peach trees they are in the range of 1–2 g per tree and per year [3]. In many cases, Fe-fertilization is done at just one or a few specific time points, for instance in the case of chelate soil applications, trunk and branch injections and foliar sprays. In other cases, the application is done on a more frequent basis and with a more diluted Fe fertilizer, such as in the case of fertirrigation.

Improving current Fe chlorosis practical correction methods will need taking into account the state-of-the-art of all related scientific knowledge, integrating physiological, biochemical and agronomical data. With this aim, we review here the recent research on Fe-fertilizers, including the development and application of new advanced analytical techniques that allow for the specific and sensitive detection of low concentrations of these Fe compounds, not only in growth media, but also in plant tissues. We also summarize and discuss the substantial basic physiological and biochemical knowledge obtained in the last years on how plants acquire, transport and utilize Fe. In all cases, Fe-fertilization leads to episodes of high Fe concentration in the rhizosphere and the roots (in cases of soil or growth substrate fertilization) or in plant shoot tissues (in cases of foliar fertilization and fertilizer injections). However, how these high-Fe episodes caused by fertilization may affect plant Fe uptake and transport processes is much less known, and this review also focuses on these poorly explored interactions.

2. Iron fertilizers

Increasing the amount of crop-available Fe has long been carried out by means of Fe-fertilizer application to soils and irrigation water, as well as to plant seeds, roots, shoots and foliage. Iron fertilizers are grouped into three main classes: inorganic Fe-compounds, synthetic Fe-chelates and natural Fe-complexes (for reviews see [2–7]).

Fertilizers based on inorganic Fe-compounds include soluble ones such as Fe salts (e.g., $Fe_2(SO_4)\cdot 7H_2O$) and insoluble

Abbreviations: BPDS, 4,7-diphenyl-1,10-phenanthrolinedisulfonic acid; *o,o*EDDHA, ethylenediamine-*N*-*N*′bis(*o*-hydroxyphenylacetic) acid; EDTA, ethylenediamine tetraacetic acid; FCR, Fe chelate reductase; MA, mugineic acid; NA, nicotianamine; PS, phytosiderophore.

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compounds such as Fe oxide-hydroxides and other cheap Fe minerals and industrial by-products [4,7]. Soluble inorganic Fe salt applications to the soil are quite inefficient, especially in high pH (i.e., calcareous) soils, due to the rapid transformation of most of the Fe applied into highly insoluble compounds such as Fe(III)-hydroxides. This occurs even when very high doses of these low-cost Fe-fertilizers are applied. Insoluble inorganic Fe-compounds have a similar prospect, and also present additional problems, such as the occurrence in many of them of other potentially toxic metals and the difficulties in matching the rates of Fe-release (from the fertilizer to the soil solution) and plant Fe uptake. Therefore, these fertilizers have a limited value as plant Fe sources, even when having low particle size and using local acidification and band application, and may cause environmental concerns [4,7]. Since high-pH Fe immobilization reactions do not occur when soluble inorganic Fe salts are applied to the foliage, they are commonly used in foliar fertilization [3,4,7]. In fact, they could provide similar or even better results in correcting Fe chlorosis than those obtained with synthetic Fe(III)-chelate fertilizers [8].

Synthetic Fe(III)-chelate fertilizers are derived from poly-aminocarboxylic acids which have high affinity for Fe(III), such as ethylenediamine tetraacetic acid (EDTA) [5–7]. These chelates are obtained by carrying out first the synthesis of the chelating agents and then incorporating Fe(III) from inorganic salts. Synthetic Fe (III)-chelates are remarkably effective as soil fertilizers, even in calcareous soils, because Fe is bound to the chelating agent over a wide range of pH values and therefore remains soluble. In the particular case of calcareous soils, synthetic Fe(III)-chelates from chelating agents with phenolic groups (e.g., the ethylenediamine-*N*-*N*′bis(*o*-hydroxyphenylacetic) acid; *o*,*o*EDDHA) are very effective Fe fertilizers. However, synthetic Fe(III)-chelates are expensive, and therefore widely used only in high-value crops such as fruit trees. Polyaminocarboxylate chelating agents used in Fe-fertilization are also under scrutiny due to their influence on metal availability and mobility, especially because of their persistence in the environment [9].

Natural Fe-complex fertilizers include a large number of substances (e.g., humates, lignosulfonates, amino acids, gluconate, citrate, etc.) from different origins, generally derived from natural products and including both polymeric and non-polymeric species [6,7,10]. Natural Fe-complex fertilizers are less stable in the soil than synthetic Fe(III)-chelates, and are easily involved in reactions of metal- and ligand-exchange and/or adsorption on soil solid phase, [10,11] thus reducing the plant-availability of the Fe delivered with the fertilizer. For this reason they are generally recommended only in soil-less horticulture and foliar applications [6,10–12].

In the last years, research on Fe fertilizers has focused on three main issues: the development of new fertilizers (including slow-release, environmentally-friendly and high purity ones), the study of application strategies and the development of new, specific and sensitive methods for Fe-compound analysis, including mass spectrometry methodologies and the use of stable isotopes. Other studies, including the combination of different classes of Fe fertilizers, are also being developed (e.g., [13,14]).

2.1. New iron fertilizers

New slow-release Fe fertilizers are water insoluble, linear chain phosphates partially polymerized, with the phosphate chain functioning as a cation-exchange backbone [15,16]. These phosphates can be solubilised by compounds with affinity for Fe, such as citrate and diethylenetriamine pentaacetic acid (DTPA). Therefore, these slow-release Fe fertilizers can be dissolved by carboxylates secreted by roots, resulting in a high Fe bioavailability. This is in line with the use of the Fe-phosphate vivianite [17].

Also, the Fe(III)-chelates of two new biodegradable, synthetic chelating agents structurally similar to EDTA, *N*-(1,2-dicarboxyethyl)-D,L-aspartic acid (IDHA) and ethylenediaminedisuccinic acid (EDDS) have been assessed as plant Fe-sources. Both chelates were successfully used as Fe fertilizers in several plant species both when applied to the foliage [18–20] and in hydroponics [19–21]. In general, these two chelates had an efficacy similar to that of Fe(III)-EDTA. However, Fe(III)-IDHA was not as effective as Fe(III)-EDTA in foliar sprays to peach trees [22], with the efficacy being dependent on the surfactant used [23]. The performance of Fe(III)-EDDS was markedly dependent of the soil pH, being more efficient in acid soils [18].

Recently, the Fe(III)-chelates of three chelating agents structurally similar to *o*,*o*EDDHA, *N*,*N*′-bis(2-hydroxy-5-methylphenyl) ethylendiamine-*N*,*N*′-diacetic acid (HJB), *N*,*N*′-bis(2-hydroxybenzyl) ethylenediamine-*N*,*N*′-diacetic acid (HBED) and 2-(2-((2-hydroxybenzyl)amino)ethylamino)-2-(2-hydroxyphenyl)acetic acid (DCHA), have also been proposed as Fe fertilizers. Iron(III)-HJB and Fe(III)-HBED were introduced because they have a much higher purity than fertilizers based on Fe(III)-EDDHA, with no optical isomers or other by-products being present [24,25], while Fe(III)-DCHA has an intermediate stability between those of Fe(III)-*o*,*o*EDDHA and Fe(III)-*o*,*p*EDDHA [26]. Although Fe(III)-DCHA is capable of maintaining Fe in soluble forms in soil solutions, its effectiveness with plants is still to be confirmed. Recently, several studies have assessed the effectiveness of Fe-compounds such as regio-isomers (e.g., Fe(III)-*o*,*p*-EDDHA), poly-condensation products and other by-products derived of the industrial synthesis procedures of the phenolic Fe(III)-chelate fertilizers. For instance, Fe(III)-*o*,*p*EDDHA has been found to be as effective as Fe(III)-*o*,*o*EDDHA in nutrient solutions [27–29], but not in calcareous soils [27,30,31], and a mixture of Fe(III)-EDDHA poly-condensate by-products was not effective in chlorosis correction [30,32].

A different approach is to promote the formation of natural Fe-fertilizers in the rhizosphere by using intercropping with grasses, which excrete Fe-chelating phytosiderophores (PS), therefore improving soil Fe solubility [2,33]. For instance, it has been proven that graminaceous cover species improve the Fe-nutrition of highly Fe chlorosis-susceptible citrus plants grown on calcareous soils [34].

2.2. New research on strategies for iron fertilizer application

Concentrating inorganic Fe-compounds in a band or spot in the soil, rather than using broadcast application, minimizes contact with soil particles, slows down processes that decrease Fe bioavailability and results in an improved fertilizer use-efficiency [4]. Similarly, the co-localization of controlled-release Fe fertilizers containing soluble inorganic Fe-compounds (e.g., $Fe_2(SO_4)$) with seeds or seedlings can improve the use-efficiency of Fe-fertilization in calcareous soils [35]. The reason behind this improvement is that the soil-fixation of the Fe released by the fertilizer is prevented by rapid root uptake. A close contact between roots and fertilizer granules is crucial, since the use of a barrier to prevent such contact decreased the leaf concentrations of Fe and other micronutrients near to critical levels and markedly reduced yield in rice [36].

On the other hand, attempts to reduce leaching of synthetic Fe (III)-chelates from the rhizosphere have been reported: Fe(III)-*o*,*o*EDDHA immobilized on *p*-nitrophenylchloroformate-activated Sepharose was effective as a soil-applied Fe-source for cucumber plants, significantly increasing cost-effectiveness and reducing environmental concerns [37,38]. Finally, the importance of using appropriate surfactants and co-adjuvants in foliar Fe fertilization has been established; appropriate foliar Fe-fertilizer formulations could be much more effective than previously thought [22,23].

2.3. New analytical methods of analysis for iron compounds

To achieve a knowledge-based correction of Fe chlorosis it is necessary to know what Fe-compounds are present in commercial fertilizers, and how they are taken up and transported in the plant. Several analytical methods capable of determining the Fe fraction chelated by synthetic chelating agents in commercial fertilizers were developed in the last two decades (for a review see [39]), mainly aimed to check fertilizer composition compliance with European directives. The application of these methods revealed a frequent disagreement between the content analyzed and that declared in the fertilizer label, with many products failing to reach the minimum amount legally required, and also the occurrence in fertilizers of a significant water-soluble Fe fraction not bound to any authorized synthetic chelating agent. In fertilizers containing phenolic synthetic Fe(III)-chelates, the amount of Fe bound to unknown compounds often accounts for 40–50% of the total [40]. Also, other compounds such as regio-isomers (e.g., *o*,*p*-EDDHA and *p*,*p*-EDDHA in fertilizers containing *o*,*o*EDDHA), poly-condensation products and other by-products, derived from impurities of the starting reagents used in the industrial synthesis procedures, have been found in fertilizers based on Fe(III)-EDDHA, Fe(III)-EDDHMA and Fe(III)-EDDHSA (see references in [39,41]). These findings led to several changes in the European directives, resulting in the inclusion of some of these compounds in the list of authorized chelating agents for micronutrient fertilizers, and also in a reduction of the threshold for soluble Fe that must be bound to authorized chelators (see references in [39]). Most of the common analytical methods are of limited usefulness, because they can determine only one or a few synthetic Fe(III)-chelates and also because of the limitations of the detection technique used (UV–visible), which results in poor limits of detection and low specificity for compounds that are chemically similar.

New advanced methodologies have been recently developed for the specific and sensitive determination of Fe(III)-chelates and chelating agents commonly used in agriculture; methods are available for irrigation water, fertilizer solutions, nutrient solutions, plant fluids such as xylem sap and plant tissues [41–43]. First, a method based on high-performance liquid chromatography-electrospray ionization-time of flight mass spectrometry (HPLC-ESI-TOFMS) allows for the simultaneous determination of the major seven ferric synthetic chetales used in Fe-fertilization: Fe(III)-EDTA, Fe(III)-DTPA, Fe(III)-HEDTA, Fe(III)-CDTA, Fe(III)-*o*,*o*EDDHA, Fe(III)-*o*,*p*EDDHA, and Fe(III)-EDDHMA [43]. Also, a new specific and sensitive method based on HPLC-ESI-TOFMS was designed to determine the amounts of the commonly used chelate Fe(III)-*o*,*o*EDDHA in plant tissues, overcoming the strong matrix effects found [42]. The method permitted to find Fe(III)-*o*,*o*EDDHA and *o*,*o*EDDHA in leaves, roots and xylem sap of tomato and sugar beet plants treated with the chelate. Furthermore, several mass spectrometry techniques have been used to obtain typical MS-MS fingerprints of the most common polyaminocarboxylate Fe(III)-chelates [41]; this information constitutes a useful tool for monitoring known active agents in plants and the environment, as well as to identify unknown impurities in commercial chelates. Other methodologies, including NMR [44] and others [45] have been developed to investigate the structural identity of the ligands and the fraction of Fe complexed in fertilizers.

2.4. Using iron stable isotopes as tracers

Another new approach to study plant Fe fertilization, uptake and translocation is the use of stable isotope tracer methodologies, which also provide the possibility to carry out long-term experiments [46]. Although radioactive Fe isotopes are still being used to evaluate the efficiency of fertilizers and to trace the Fe applied within the plant (e.g., [34,47]), the use of stable Fe isotopes (mainly ^{57}Fe) as Fe tracers is increasing [25,27,29,48]. Recently, a dual-stable Fe isotope tracer method has been used to study the uptake, xylem transport and distribution of Fe and chelating agents from two different Fe(III)-*o*,*o*EDDHA stereoisomers applied simultaneously to Fe-deficient plants [48]. The usefulness of this method in plant Fe nutrition has been proven, since no short-term Fe isotope exchange reactions occurred with the Fe(III)-chelates used.

These combined analytical methods have shed light on some previously unknown physiological processes that should be taken in consideration when studying the physiology and biochemistry of plants affected by Fe-deficiency. For instance, it has been clearly established that in plants fertilized with Fe(III)-*o*,*o*EDDHA both the chelating agent and the chelate are present in appreciable concentrations in all tissues analyzed [42,48]. This could be of critical importance when studying Fe-homeostasis mutants (see below).

3. Root iron uptake mechanisms

3.1. Reduction- and chelation-based iron uptake strategies

Plants use two mechanisms for Fe uptake from the growth media (soils, inert substrates, nutrient solutions, etc.): a reduction-based strategy (Strategy I) and a chelation-based one (Strategy II), the latter restricted to grasses [49]. Many of the molecular components of both strategies have been elucidated in the last two decades, whereas other aspects such as signaling pathways, the identity of possible Fe sensors in regulatory mechanisms and the physiological interplay of the individual components still remain unclear. Iron uptake mechanisms in Strategy I plants also involve marked metabolic [50] and morphological [51] changes.

The core components of the reduction-based mechanism are an Fe-reductase enzyme that belongs to the FRO (Ferric Reductase Oxidase) family and an IRT (Iron Regulated Transporter) Fe(II) transporter that belongs to the ZIP (ZRT, IRT-like protein) family (see recent reviews [52–55]) (Fig. 1). Both components are regulated by the Fe-inducible FIT/FER (FER-Like Iron deficiency-induced Transcription factor) transcription factor [56], which forms heterodimers with other basic helix-loop-helix (bHLH) transcription factors [57–59]. Two new transcription factors involved in Fe homeostasis, POPEYE (PYE) and BRUTUS (BTS), have been recently reported [60]. Also, IRT2 is involved in intracellular Fe trafficking in Fe-deficient roots [61]. Additional elements associated with the root reduction-based mechanism include the excretion to the rhizosphere of protons and of a plethora of organic compounds, including carboxylates, phenolics, and flavonoids, which can affect Fe-availability directly or indirectly [62,63] (Fig. 1). The acidification process relies on a Fe-inducible proton extrusion pump of the AHA family (*Arabidopsis* H^+ ATPase), which lowers the pH of the growth media, facilitating the solubilization of inorganic Fe [64–67]. However, the information on the transporters and mechanisms of accumulation and excretion of organic compounds as well as their roles is still scarce. Members of the MATE (Multidrug And Toxic compound Extrusion) and ABC (ATP-Binding Cassette) families of transporters are likely involved in this organic compound trafficking [62]. PEZ1 (Phenolics Efflux Zero), a member of a MATE subfamily, is induced by Fe, localized in the plasma membrane and is involved in the root cell efflux of protocatechuic (3,4-dihydroxybenzoic) and caffeic acids [68]. AtPDR9, a member of the PDR (Pleiotropic Drug Resistance) subfamily of ABC transporters, is expressed predominantly in the lateral root cap and epidermal cells at the root tip [69] and while its exact role in Fe homeostasis remains elusive, it may be involved in the export of phenolic

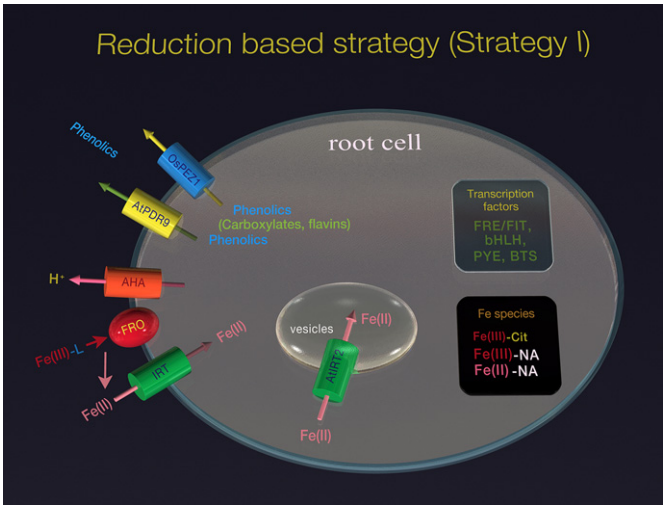

Fig. 1. Reduction-based (Strategy I) root Fe uptake responses to Fe-deficiency. The Fe (III) reductase enzyme (FRO) can work with different Fe(III)-compounds (Fe(III)-L, where L is an organic ligand with affinity for Fe), and the resulting Fe(II) is taken up by the IRT transporter. Once into the cell, Fe(II) can be transported to vesicles by another Fe(II) IRT transporter. An ATPase (AHA) excretes protons to the apoplast, and different transporters excrete phenolics (AtPDR9 and OsPEZ1), carboxylates, flavins and other compounds. The system is regulated by several transcription factors (FRE/FIT, bHlHs, PYE, BTS). Also, several Fe species may occur in the cytoplasm, including Fe(III)-NA and Fe(II)-NA and Fe(III)-citrate, the last one being less likely.

compounds such as caffeic or chlorogenic acids, which may improve the reutilization of apoplastic Fe [70,71].

In grasses (Strategy II plants), natural Fe(III) chelators such as the mugineic acid (MA) family of PSs are used to acquire sparingly soluble Fe from the rhizosphere (Fig. 2). The response of the chelation-based mechanism plants to Fe-deficient conditions includes the biosynthesis and secretion of PSs to the rhizosphere and the subsequent uptake of Fe(III)-PS complex(es). Many molecular components of this mechanism have been elucidated in recent years (Fig. 2). Phytosiderophores are synthesized from methionine and the components of this biosynthetic pathway show

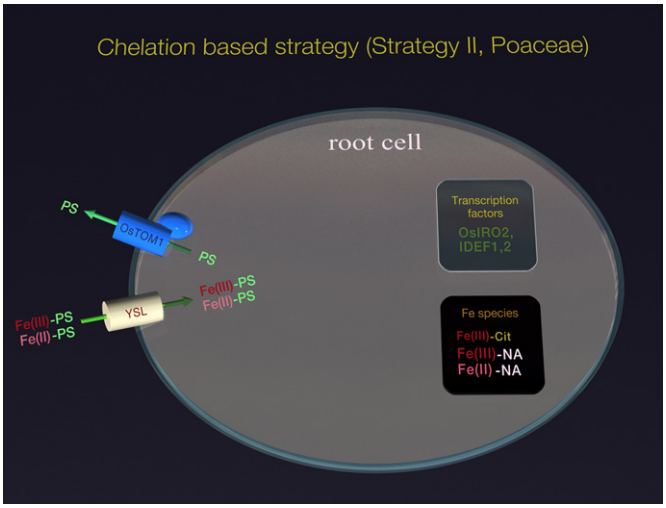

Fig. 2. Chelation-based (Strategy II) root Fe uptake responses to Fe-deficiency in grasses. Phytosiderophores (PS) are excreted by transporters (OsTOM1) or vesicle exocytosis, and after recruiting Fe from the growth media Fe-PS complexes are taken up by YSL transporters. The system is regulated by transcription factors (IDEF1, 2, OsIRO2). Several Fe species may occur in the cytoplasm, including Fe(III)-NA and Fe(II)-NA and Fe(III)-citrate, the last one being less likely.

strongly enhanced expression under Fe deficiency, resulting in an increased PS release [72]. The first putative PS plasma membrane transporter, TOM1 (Transporter Of MA), has been recently described; *TOM1* is up-regulated by Fe deficiency and plants over-expressing TOM1 excrete more PSs without increasing the rate of synthesis [73]. The subsequent uptake of Fe(III)-PS from the rhizosphere is mediated by YS1, that was first isolated in *Zea mays* [74,75]. Electrophysiological analyses revealed that YS1 functions as a proton-coupled symporter for various MA-bound metals, including Fe(III), Zn(II), Cu(II), and Ni(II) [76]. A barley homolog of *YS1* (*HvYS1*) was isolated and shown to transport Fe(III)-MA [77]. Some components of the signaling mechanism in grasses are well known (see recent review [59]): two *cis* elements (IDE1 and IDE2; Iron-Deficiency-responsive Elements 1 and 2) confer gene Fe deficiency-inducible expression [78] and recently, *trans*-factors binding each of these elements (IDEF1 and IDEF2; IDE-binding Factor 1 and 2) were also identified [79,80]. Another transcription factor, *OsIRO2* (iron-related transcription factor 2), regulates the PS-mediated Fe uptake system in rice [81,82].

The reduction- and chelation-based strategies are not mutually exclusive, since in rice and barley Fe-deficiency induces uptake of both Fe(II) -by IRT1- and Fe(III)-PS [83,84]; however, rice apparently cannot carry out Fe(III) reduction [85], suggesting that the expression of IRT1 may be a particular adaptation to soil conditions existing in flooded rice soils, where Fe(II) levels are high [59]. Also, the fact that the transporter AtYSL3 (Yellow Stripe-Like 3) is up-regulated in Fe-deficient *Arabidopsis* roots [86] may suggest that direct uptake of some Fe-compounds could also occur in Strategy I plants.

3.2. Root iron fertilization and plant iron uptake mechanisms

Although components of both strategies are well characterized at the molecular level, physiological aspects as important as their response to Fe fertilization have been less explored. When soluble Fe-fertilizers are applied to the soil, Fe concentrations in the rhizosphere would reach rapidly values from 50 μM to 10 mM, depending on the fertilization application technique used. Although with these relatively high Fe concentrations a down-regulation of the molecular components involved in root Fe uptake would be expected, the real effects are still unexplored. Moreover, this could occur several times during the growth season, every time fertilizer is applied, with the “high-Fe” event lasting for days or even weeks.

Root Fe-fertilization leads to a de-activation of at least some root Fe acquisition mechanisms. Upon Fe resupply to Fe-deficient tobacco plants, the root transcript accumulation of *FRO* and *IRT* decreased to control values within 24 h [87]. In sugar beet, the root Fe chelate reductase (FCR) activity was reported to decrease more slowly with Fe-resupply, with a 20% decrease in 24 h, to go down to control levels in 96 h [88]. In cucumber, a considerably lower capacity to decrease the pH of the nutrient solution was also observed upon Fe-resupply [67]. Other reduction-based strategy components also show a rapid down-regulation; for instance, the transcript abundance of 6,7-Dimethyl-8-ribityllumazine synthase (DMRLs), an enzyme involved in Riboflavin (Rbfl) synthesis, decreased rapidly, whereas the amounts of the corresponding protein (DMRLs) and metabolite (Rbfl) remained constant [50]. This down-regulation of root responses is markedly different to what occurs in leaves, since leaf *FRO* and *IRT* transcript levels increase 24 h after fertilization in tomato [89] and FCR activity increases 20% in sugar beet [90]. On the other hand, it must be taken into account that the root FCR activity needs some Fe: it has been often reported that root FCR activity is higher when the concentration of Fe in the growth media is in the range 0.3–2.0 μM than when Fe

concentrations are in the range 0–0.1 μM [91–93]. In some cases, a transient increase in root FCR activity occurs upon Fe-resupply to Fe-deficient plants, with FCR decreasing gradually with time towards rates present in Fe-sufficient conditions [93,94].

Recent studies indicate that upon Fe-fertilization significant amounts of Fe(III)-chelates and chelating agents could enter the plant [42,48]. When Fe-deficient sugar beet plants were resupplied with two Fe(III)-chelate isomers, FCR rates, xylem transport and total uptake were 2-fold higher with the *meso* isomer than with the *racemic* one, but both chelating agent isomers were incorporated and distributed by plants at similar rates, in amounts one order of magnitude lower than those of Fe. Most of the Fe acquired was localized in roots, whereas most of the chelating agent was localized in leaves. Although most of the Fe was taken up by the plant through a dissociative reduction mechanism, a small part of the Fe delivered by the Fe(III)-*o*,*o*EDDHA could had been taken up *via* non-dissociative mechanism(s), probably using the transpiration stream as the driving force for entry. These uptake mechanisms may be relevant in the short term after Fe fertilization and also whenever root FCR activity is down-regulated [48].

3.3. Iron chelate reductase assay: a questionable method

The measurement of FCR rates with assays using ligands with high affinity for Fe(II), such as BPDS and ferrozine, was initially designed to prove the existence of a reduction-based strategy [95]. This methodology has later become a common and useful tool to assess FCR rates obtained with different species and genotypes in standard conditions and test Fe-fertilizer efficiencies. However, there are significant concerns about this methodology. The strong affinity of the chelating agents for Fe(II) could fully displace the reaction equilibrium [96], leading to full-blown FCR activities, much higher than those occurring *in vivo*. Also, BPDS has non-negligible affinities for Fe(III) [97], may form ternary complexes Fe(III)/Fe(III) chelator/BPDS [98] and Fe(II)-BPDS could be adsorbed by plant roots. Another constraint when using FCR rates to assay fertilizer efficiency is the poor knowledge of the catalytic mechanism and substrate specificity of the Fe reduction/uptake process. It has been reported that strong chelating agents could chelate the Fe in the FCR heme group, therefore decreasing enzymatic activities [99].

Some studies have shown that the amount of Fe reduced is considerably higher than the amount taken up by the plant [48,100,101]. This overestimation could be due to the BPDS affinity for Fe(II) or other unknown aspects of the catalytic mechanism. Also, there is no explanation so far for the fact that FCR rates are markedly different with chelate isomers such as *racemic* and *meso* Fe(III)-*o*,*o*EDDHA [48,101].

Therefore, it is necessary to focus on the development of new assays based in monitoring directly the decreases in the concentrations of the enzyme substrates or increases in enzyme products, including Fe(III)- and Fe(II)-chelates and Fe(II).

4. Long distance iron transport

Once Fe is acquired by root epidermal cells, it is likely transported symplastically to the pericycle cells, and then to the vascular cylinder and the xylem stream.

4.1. Xylem iron loading

The knowledge on Fe xylem loading is still scarce and many questions are still open, the major ones being the chemical form(s) and mechanism(s) involved in Fe loading (Fig. 3). The transporter that loads Fe into the xylem is not yet known, although it is

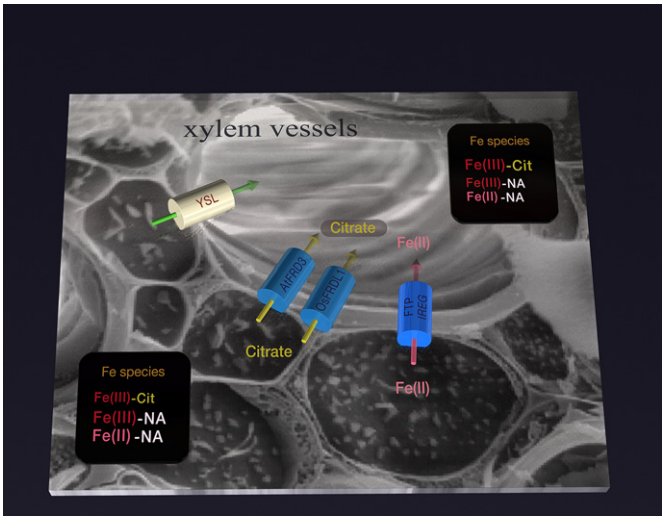

Fig. 3. Xylem Fe loading in plants. Citrate is loaded by MATE-family transporters (AtFRD3, OsFRDL1). Iron can be loaded by ferroportins (FTP/IREG), and may be also loaded as Fe-complexes by transporters of the YSL family. Several Fe species may occur, with Fe-NA complexes being more likely in the cytoplasm, and Fe(III)-citrate being more likely in the xylem sap.

commonly accepted that at the neutral pH found in the cytosol Fe is probably chelated with the non-proteinogenic amino acid nicotianamine (NA) [102,103]. Therefore, the Fe(III)-NA complex itself could be loaded into the xylem by an Fe-PS transporter from the YSL (Yellow Stripe-Like) family. A possible candidate is AtYSL2, which is expressed in lateral membranes of xylem parenchyma cells, thus suggesting a role in lateral movement within the veins; however, there are contradictory results about the ability of AtYSL2 to transport Fe-NA [104,105]. The rice ortholog OsYSL2 also localizes to the lateral plasma membrane and transports Fe-NA, although it has been proposed to be involved in phloem transport [106]. Another Fe transporter, IREG1/FPN1 (Iron Regulated1/Ferroportin1) has also been proposed to play a role in Fe xylem loading, since the loss of *FPN1* results in chlorosis and FPN1-GUS plants show staining in the plasma membrane in the root vasculature [107]. However, FPN1 yeast complementation studies have failed, and no information on the chemical form of Fe transported by FPN1 is yet available [107].

Carboxylates may also have a role in xylem Fe loading (Fig. 3). Citrate has been described as an Fe(III) chelator in the xylem sap [108], and FRD3 (Ferric Reductase Defective), a transporter of the MATE family, localizes to the plasma membrane of the pericycle and the vascular cylinder. FRD3 proteins carry out citrate efflux into the root vasculature and have been described in *Arabidopsis* [109], rice [110] and rye [111]. Mutant *frd3* plants are chlorotic, show reduced citrate and Fe concentrations in the xylem and shoots, accumulate Fe in the root and exhibit constitutive expression of the Fe uptake responses, therefore suggesting that FRD3 is necessary for efficient Fe translocation to the shoot [109,110,112,113]. However, an alternative explanation for the impaired Fe homeostasis in *frd3* plants is that the xylem C transport carried out by FRD3 in the Fe-deficient wild type may indirectly boost root Strategy I responses. Also, independent Fe-citrate and Fe-NA xylem loading systems may complement each other, since in the *Arabidopsis frd3* mutant the nicotianamine synthase NAS4 gene is induced, and the double mutant *nas4x-2*/*frd3* shows impaired growth and low shoot Fe concentrations [114].

Xylem Fe loading studies should take into account the recent finding that in plants fertilized with synthetic chelates both the chelating agent and the chelate could be present in appreciable concentrations in plant tissues [42,48]. Therefore, systems using

natural Fe-compounds (e.g., Fe-citrate) in the growth media should be preferred in Fe homeostasis studies. For instance, knock-out transporter mutants could, if grown with Fe(III)-chelates, still transport significant amounts of Fe even when native Fe forms are no longer transported.

4.2. Xylem iron transport

Iron is assumed to be transported in the xylem as a complex form, because free Fe ionic forms can be toxic and are also prone to undergo precipitation at the neutral or slightly acidic pH values typical of xylem sap. Major developments have been reported in the field of analysis of natural Fe compounds in plants. Nicotianamine has six functional groups that allow octahedral coordination, and the distances between functional groups are optimal for the formation of chelate rings; accordingly, NA chelates many metals and forms stable complexes with both Fe(II) and Fe(III) at neutral and weakly alkaline pH values [102,103,115]. Nicotianamine has been proposed to play major roles in symplastic and phloem Fe chelation, whereas its possible role in long-distance metal transport in the xylem is still being explored ([116]; see [75] for a review). Although NA has been observed in the xylem at μM concentrations [117], NA-Fe chelates have not been detected in xylem sap so far. Nicotianamine does not seem to be essential for xylem Fe transport since the NA-deficient tomato mutant *chloronerva* accumulates Fe in old leaves [118]. The possibility that Fe could be chelated to PS in the xylem sap of Strategy II species is still an open question.

Citrate has been considered for many years as the most likely candidate for Fe xylem transport [119–121], but the identity of Fe-Cit complexes in the xylem sap had only been hypothesized by means of *in silico* calculations using total concentrations of possible Fe complexing agents (including carboxylates) and Fe, and the known stability constants of Fe-containing complexes, always assuming that chemical equilibrium was achieved. Using this approach, several Fe-Cit species were predicted to be the most abundant Fe complexes in the xylem sap whereas other potential plant metal chelators such as NA were ruled out as possible xylem Fe carriers [102,103]. A tri-Fe(III), tri-citrate complex (Fe_3Cit_3) was recently found in the xylem sap of Fe-deficient tomato resupplied with Fe, using an integrated MS approach based on exact molecular mass, isotopic signature, Fe determination and retention time [108]. The complex was modeled as having an oxo-bridged tri-Fe core [108]. A second complex, the binuclear Fe(III)-Cit species Fe_2Cit_2, was also detected in Fe-citrate standards along with Fe_3Cit_3, with the allocation of Fe between the two complexes depending on the Fe to citrate ratio. Since plant xylem has a wide range of Fe to citrate ratios, both species could occur in different conditions [108]. However, the Fe_3Cit_3 complex has only been found so far in Fe-resupplied plants, which have Fe concentrations above the limit of detection (approximately 20 μM), whereas the complex could not be detected in Fe-deficient and control plants, which have lower xylem sap Fe concentrations. It is also possible that other Fe-complexes could exist in some conditions, perhaps involving other major carboxylates present in the xylem sap and/or NA.

4.3. Xylem iron unloading

Iron is unloaded from the vasculature into leaf tissues through yet unknown mechanisms. These processes could take place *via* parenchyma cells and/or by passive diffusion to the apoplastic space driven by transpiration. The symplastic path could imply the participation of transporters of the YSL family as well as a reduction-based mechanism. Several YSL, FRO and ZIP proteins have been localized in the vascular cylinder and might play a role in Fe unloading [53,55,75,86].

On the other hand, there is evidence that the xylem system is affected in chlorotic leaves, since xylem vessels are smaller and the vascular bundle is disorganized and heterogeneous in size and shape [23,122]. These morphological alterations may drive Fe towards symplastic or apoplastic unloading paths different than those present in Fe-sufficient plants, and these changes in unloading mechanisms may potentially lead to subsequent Fe precipitation (see below). Determining the precise localization of leaf Fe accumulation sites would be crucial to unravel these questions.

4.4. Phloem iron transport

At least two possible Fe forms could occur in the phloem. The first one is a complex with NA, since the neutral to basic pH values of the phloem sap are suitable for Fe-NA formation [102,103]. YSL transporters able to transport Fe-NA complexes have been described in *Arabidopsis* and rice phloem vascular tissues (see [75] for a review); some of these transporters could facilitate xylem–phloem Fe exchange. Also, a protein capable to bind Fe, ITP (Iron Transport Protein), was described in the phloem sap of *Ricinus communis* L. plants [123].

4.5. Iron fertilization and plant iron transport mechanisms

Only few studies have been made on the interaction between Fe fertilization and Fe long-distance transport, in spite of the fact that injection of Fe salts (mainly Fe(II)-sulphate and Fe ammonium citrate) in trunks and branches, both in liquid and solid formulations, have been reported to alleviate Fe chlorosis in different tree species [2,124]. The scientific rationale for these practices is now clear, since Fe(III)-citrate complexes have been found in the xylem of Fe-deficient plants resupplied with different Fe forms *via* root applications, indicating that plants can withstand relatively high concentrations of Fe(III)-citrate while transporting Fe efficiently to the shoots [108]. The fact that high-stability synthetic Fe(III)-chelates are also effective when injected to the trunk of Fe-deficient trees (see references in [2]) indicate that the unloading system can also work with a wide range of Fe(III)-compounds.

However, especially when using liquid injection applications, there is always a risk of causing phytotoxicity in shoots and leaves when Fe concentration, application and timing are not properly chosen. Acquiring more knowledge on the natural chemical forms of Fe in xylem sap and the dynamics of Fe loading and unloading would be critical to improve fertilization techniques, especially trunk and branch injections, in the future. The available evidence supports that Fe(III)-citrate could be directly used as an Fe-fertilizer, although the possible effects of shoot fertilization on the root Fe-deficiency response mechanisms are fully unexplored.

5. Iron in leaves

5.1. Iron uptake by leaf mesophyll cells

The mechanism(s) by which Fe is acquired by leaf mesophyll cells possibly includes an FCR enzyme and an IRT transporter, although the process is not as well known as those operating in roots (Fig. 4). An FCR activity is present in leaf tissues, but it is not clearly up-regulated upon Fe-deficiency, conversely to what occurs in root cells [125]. Also, whereas a light-dependent FCR activity is present in excised leaf disks [126–129], it is complex to discriminate the true cell FCR activity from Fe(III) reduction activities arising from exposed organelles in broken cells at the leaf disk

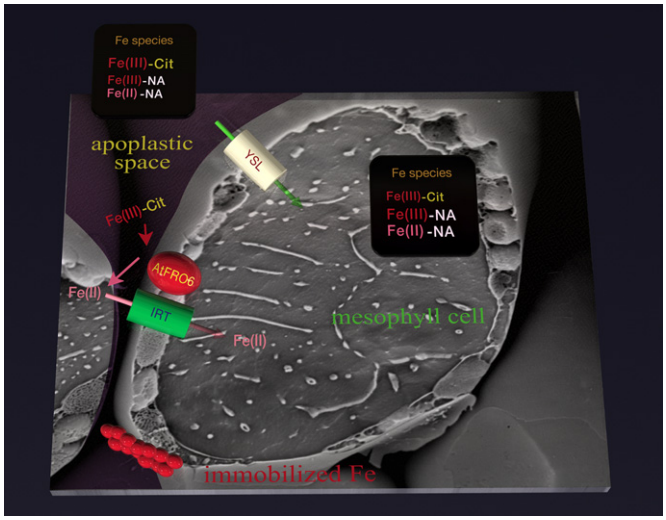

Fig. 4. Iron uptake by leaf mesophyll cells. An Fe(III) reductase enzyme (AtFRO6) can work with Fe(III)-citrate (Fe(III)-Cit) and perhaps other Fe(III)-complexes, and the resulting Fe(II) is taken up by an IRT transporter. Other YSL transporters can also participate in cell Fe uptake. Several Fe species may occur, with Fe(III)-citrate being more likely in the apoplast and Fe-NA complexes being more likely in the cytoplasm. Iron can be immobilized in inactive forms in the apoplast.

edges and that caused by excretion of reducing compounds [125]. A light-dependent FCR activity was also characterized using isolated protoplasts, and the lack of enhancement of FCR activity upon Fe-deficiency was linked to an insufficient reducing power in Fe-deficient cells [126]. Factors such as differences in apoplastic pH and carboxylate concentrations between Fe-deficient and Fe-sufficient plants may also regulate leaf FCR activity, and in that regard the identity of the physiological substrates for this enzyme is still a key open question.

In *Arabidopsis*, *FRO* and *IRT*-like genes are expressed in leaves [53–55,130], and for instance FRO6 is localized in the leaf plasma membrane [131] and protoplasts prepared from *fro6* plants show reduced FCR activity [54]. However, *fro6* mutant plants do not display any obvious phenotype [54], suggesting that FRO6 may function redundantly with another FRO family member or with another reduction mechanism. An alternative explanation is that FCR activity may be not essential for Fe uptake by leaf cells. The expression of FRO6 is also light-inducible [54].

Light has also been proposed to directly photo-reduce Fe(III)-citrate complexes in the leaf apoplast, therefore facilitating Fe(II) uptake into the leaf cell symplast by IRT transporters [128]. New MS-based analytical tools available would permit to test that hypothesis.

Other Fe uptake transporters may also be involved in leaf cell uptake, including several members of the YSL family that are expressed in leaves (such as AtYSL1, AtYSL3, AtYSL2, OsYSL2 and OSYSL15), although their expression is usually confined to the vascular tissue [75]. The specificity of some of these transporters with the possible substrates (Fe(III)-PSs, Fe(III)-NA or Fe(II)-NA) is still not fully known [75,105,106,132–134].

Finally, we should keep in mind that Fe can also take the symplastic route as shown by feeding studies [135], with plasmodesmata (Fig. 5) facilitating extensive communication between leaf cells.

5.2. Iron immobilization in leaves

It has been known for decades that Fe-deficient leaves may have Fe concentrations similar or even higher than those present in Fe-sufficient leaves. This has been termed the "Fe-chlorosis paradox"

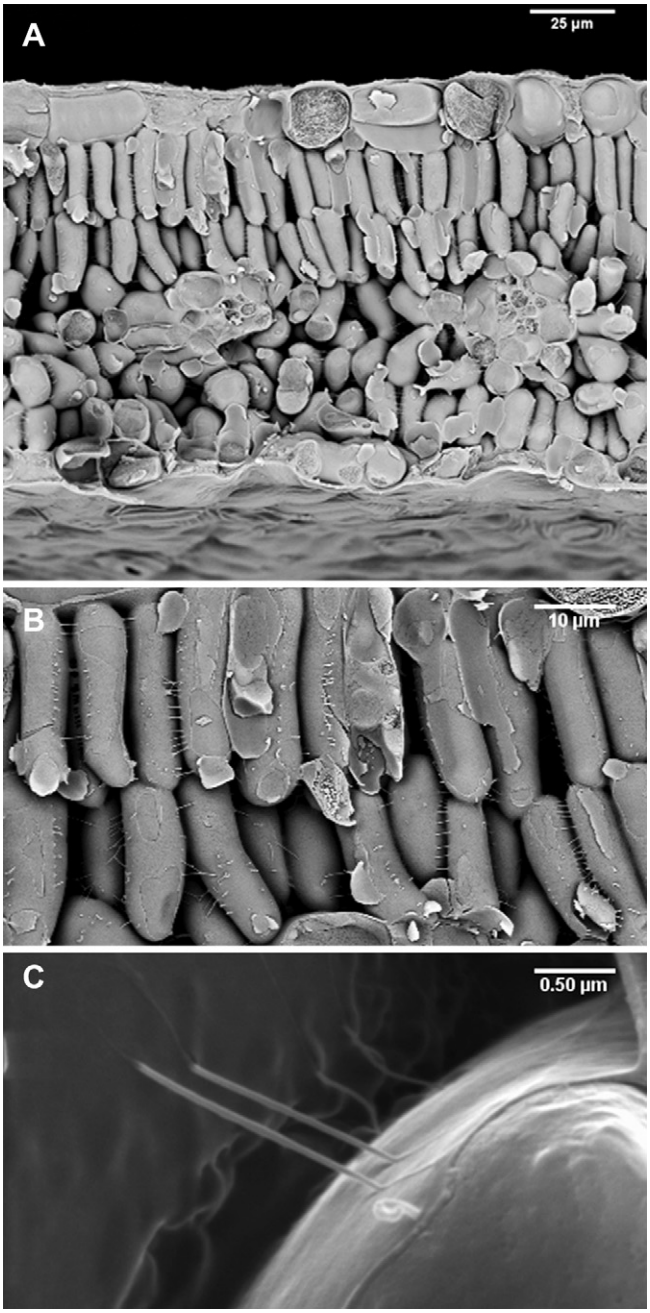

Fig. 5. Plasmodesmatal connection between leaf mesophyll cells. Images of: a whole peach (*Prunus persica* (L.) Bastch) leaf cross-section (A), a zoom showing cells connected by plasmodesmata (B) and a higher magnification close-up of the same cells (C). Peach trees were grown in calcareous soils under field conditions. Micrographs were taken with low temperature-scanning electron microscopy (LT-SEM).

[136,137]. Iron-deficient leaves of peach [138] and tomato [89] accumulate more Fe in the midrib and veins, with Fe concentrations being markedly lower in mesophyll areas; this was shown using two-dimensional nutrient mapping obtained by illuminating leaf surfaces perpendicularly with synchrotron X-ray radiation and measuring fluorescence. Whereas these studies support the occurrence of Fe immobilization in areas close to the vascular system in Fe-deficient leaves, more detailed leaf cross-section analysis using high-resolution image techniques would be needed to ascertain the exact localization of these Fe pools. Preliminary data obtained with Perl's staining suggest that Fe is indeed in high concentrations in vascular tissues of Fe-deficient leaves (Fig. 6).

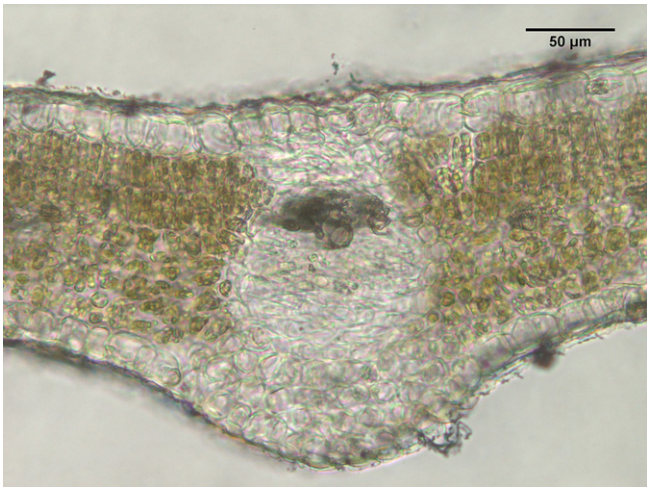

Fig. 6. Cross-section micrograph of an Fe-deficient peach (*Prunus persica* (L.) Bastch) leaf, stained with diaminobenzidine-enhanced Perls' staining. Peach trees were grown in calcareous soils in field conditions. The black color appearing in vascular tissues indicates a high concentration of Fe in this area.

The forms of Fe accumulation in tissues and cells are still unclear. The actual chemical form of Fe in leaf immobilized pools of Fe-deficient plants has been hypothesized to consist in phosphate-oxide Fe compounds, but little is known about its nature so far. On the other hand, upon Fe-resupply Fe is transported rapidly to the chloroplast and then stored in ferritins, proteins that can hold a large number of Fe atoms [139]. Iron can be later re-mobilized from ferritins towards Fe sinks.

5.3. Iron uptake by cell organelles

Photosynthesis and respiration, two of the most important cell functions where Fe plays a role, take place inside the subcellular organelles chloroplast and mitochondria. However, the mechanisms of Fe delivery to its final targets in these compartments, as well as the internal Fe homeostasis processes, are not as well understood as those for other metals such as Cu, and only scattered information is available [140–142]. In the chloroplast, there are indications that Fe(III) reduction *via* FRO family members may occur; indeed FRO7 is required for uptake into the chloroplast [131]. In this compartment, Fe transport can be mediated by the permease PIC1 (Permease in Chloroplast 1), which localizes to the inner chloroplast envelope and is critical for chloroplast development [143]. MAR1 (Multiple Antibiotic Resistance 1), a homolog of IREG1, has also been proposed to transport Fe-NA into the chloroplast [144,145]. Recently, another chloroplastic transporter, ZmFRD3, has been localized in the thylakoids and described to transport Fe by functional complementation in yeast [146,147]. On the other hand, AtYSL6 has been recently described to act as Fe effluxer in the chloroplastic envelope of *Arabidopsis* [148].

In mitochondria, the Fe importer MIT1 (Mitochondrial Iron Transporter 1) is essential for plant growth and development [149,150] and the ABC transporter STA1 (STARIK 1)/AtATM3 (ATP-binding cassette Transporters of Mitochondria 3) has been implicated in the export of Fe-S clusters [151]. Also, *AtFRO3* and *AtFRO8* contain mitochondrial-targeting sequences and are mainly located in roots and shoot veins, respectively [53], suggesting that a reduction-based uptake could also take place in this compartment. The oxidizing conditions found in the mitochondrial intermembrane space [152] would also point to the need for a reduction-based mechanism.

Although most of the Fe is located in the chloroplast, the vacuole is essential for Fe redistribution in early developmental stages. Iron is known to be imported into the vacuole by VIT1 (Vacuolar Iron Transporter 1) [153] and IREG2 [154], and exported out to the cytoplasm by NRAMP3 and NRAMP4 (Natural Resistance-Associated Macrophage Protein) [155]. Other Fe transporters, AtYSL4 and AtYSL6, have also been located in the tonoplast in a proteomic *Arabidopsis* study, but their functions are still unknown [75,156]. Knowledge of the response of vacuolar Fe homeostasis upon fertilization techniques such as seed treatments with Fe will help developing more effective targeted strategies to prevent Fe deficiency at early stages.

5.4. Iron fertilization and leaf iron uptake

Foliar fertilization with inorganic (e.g., $FeSO_4$) and organic Fe-compounds, including natural (e.g., Fe(III)-citrate, Fe(III)-lignosulfonates, etc.) and synthetic ones (e.g., Fe(III)-DTPA, Fe(III)-EDTA, etc.) can alleviate Fe-deficiency, although in fruit tree crops this practice is still not very common and is only considered to be a valuable complement to soil fertilization [3,8]. There are two major drawbacks to develop effective spray formulations for agricultural purposes: the limited knowledge on leaf mesophyll Fe uptake mechanisms (discussed previously) and the limited understanding of the factors involved in the penetration, translocation, and bioavailability of leaf-applied Fe fertilizers [8]. The performance of Fe-sprays is affected by many factors, including plant-related, environmental and physico-chemical ones, which are not fully understood [8] and problems of reproducibility and interpretation of results from foliar Fe application studies have been described [157].

Specific Fe-staining of leaf cross-sections indicates that Fe-fertilizers can deeply penetrate leaves when applied with an appropriate surfactant (Fig. 7). The Fe applied can be distributed to a large part of the leaf internal apoplast volume, suggesting that this Fe woud be available for subsequent mesophyll cell uptake. Research should focus on the Fe forms present in the apoplast of Fe-fertilized leaves, as well as on the mobility of the Fe incorporated by fertilization. An oxidizing environment and the presence of light

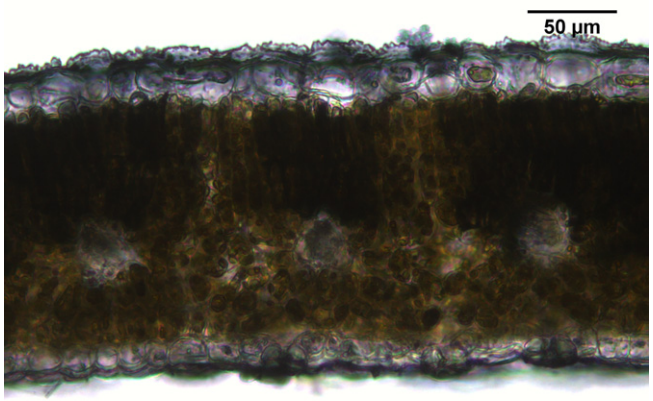

Fig. 7. Cross-section micrograph of an Fe-deficient, peach (*Prunus persica* (L.) Bastch) leaf after foliar Fe-fertilization, stained with diaminobenzidine-enhanced Perls' staining. Peach trees were grown in calcareous soils in field conditions. Foliar fertilization was carried out by briefly (2 s) immersing leaves in 2 mM $FeSO_4$ supplemented with 0.1% BreakThrough S-233 surfactant (an organo-silicon compound, polyether-modified polysiloxane, from Goldschmidt GmbH, Essen, Germany). The black color marks high Fe concentrations, indicating that Fe has penetrated in the whole leaf volume.

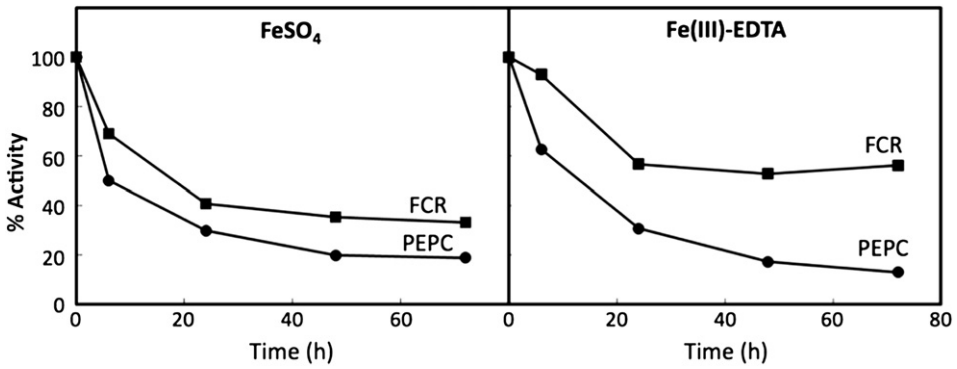

Fig. 8. Deactivation of root Fe-deficiency responses with foliar Fe fertilization. Percentage of initial phospho*enol*piruvate carboxylase (PEPC; circles) and Fe-reductase (FCR; squares) activities in root extracts from sugar beet (*Beta vulgaris* L.) Fe-deficient plants resupplied with Fe. Measurements were done at different time-points after a single Fe foliar spray with 2 mM $FeSO_4$ or Fe(III)-EDTA. Data are means ± SD of 9 plants used as replicates.

may cause Fe immobilization when foliar fertilization is applied, ultimately lowering treatment efficacy. The use of high-resolution image techniques should clarify this hypothesis.

There is also evidence that foliar Fe-fertilization could de-activate plant root Fe uptake mechanisms. In tobacco plants, a foliar spray of 100 μM Fe(III)-EDTA caused, 24 h after treatment, a decrease in root expression of *NtFRO1* and *NtIRT1* gene transcript levels to values similar to those found in control plants, whereas a lower Fe concentration (10 μM) did not cause such effects [87]. In Fe-deficient sugar beet plants, a foliar Fe application of 2 mM Fe (III)-EDTA or $FeSO_4$ (this is a commonly used Fe concentration in foliar sprays that can be very efficient in field conditions) caused decreases in root FCR activities of 10–30 and 40–65% at 6 and 24 h after application, respectively, with the decreases being larger for $FeSO_4$ (Fig. 8). The decrease upon Fe-fertilization was even larger for the activity of root phospho*enol*pyruvate carboxylase, another enzyme elicited by Fe-deficiency (Fig. 8).

All these data indicate that foliar fertilization may de-activate root Fe-reduction strategy responses very rapidly, suggesting that root responses are down-regulated directly by an Fe-dependent signal perhaps Fe itself-, possibly *via* phloem. Therefore, it should be carefully assessed whether foliar fertilization management techniques could have a deleterious effect by de-activating root responses to Fe deficiency.

6. Concluding remarks

For the optimization of the Fe-fertilization strategies it will be crucial to further improve the basic knowledge on the long and short-transport of Fe, xylem loading and unloading, Fe immobilization and the Fe acquisition processes by mesophyll leaf cells and subcellular compartments.

Iron trafficking within the plant involves the passage through many environments with different pH values and chemical composition. This implies that Fe should change from one to another chemical specie(s) in each of the corresponding environment interfaces. Furthermore, in each environment Fe could be in several different forms, and we are only starting to unravel their identity and localization by using advanced analytical technologies.

Also, it is often assumed that Fe contained in Fe-fertilizers would be taken up, transported and utilized following mechanisms and processes present in Fe-deficient plants. However, there is emerging evidence that Fe-resupply caused by Fe-fertilization could change the physiology and biochemistry of these Fe-deficient plants. Upon Fe-fertilization (either to the roots or the shoots), some of the mechanisms elicited by Fe-deficiency will be modulated or de-activated in the short term. Furthermore, some Fe-fertilizers such as Fe(III)-chelates could enter the plant directly, without using known uptake pathways. Therefore, comprehensive studies on the physiology of Fe-resupplied plants, including the effects of the different kinds of Fe-fertilization on the modulation of the reduction-based and chelation-based Fe acquisition strategies, are highly needed.

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