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Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph



Review

Discovering the role of mitochondria in the iron deficiency-induced metabolic responses of plants

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ARTICLE INFO

Article history:

Received 16 June 2011
Received in revised form
14 September 2011
Accepted 14 September 2011

Keywords:

Energy metabolism
Fe deficiency
Tricarboxylic acid (TCA) cycle
Mitochondria
Respiratory chain

ABSTRACT

In plants, iron (Fe) deficiency-induced chlorosis is a major problem, affecting both yield and quality of crops. Plants have evolved multifaceted strategies, such as reductase activity, proton extrusion, and specialised storage proteins, to mobilise Fe from the environment and distribute it within the plant. Because of its fundamental role in plant productivity, several issues concerning Fe homeostasis in plants are currently intensively studied. The activation of Fe uptake reactions requires an overall adaptation of the primary metabolism because these activities need the constant supply of energetic substrates (i.e., NADPH and ATP). Several studies concerning the metabolism of Fe-deficient plants have been conducted, but research focused on mitochondrial implications in adaptive responses to nutritional stress has only begun in recent years. Mitochondria are the energetic centre of the root cell, and they are strongly affected by Fe deficiency. Nevertheless, they display a high level of functional flexibility, which allows them to maintain the viability of the cell. Mitochondria represent a crucial target of studies on plant homeostasis, and it might be of interest to concentrate future research on understanding how mitochondria orchestrate the reprogramming of root cell metabolism under Fe deficiency. In this review, I summarise what it is known about the effect of Fe deficiency on mitochondrial metabolism and morphology. Moreover, I present a detailed view of the possible roles of mitochondria in the development of plant responses to Fe deficiency, integrating old findings with new and discussing new hypotheses for future investigations.

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Introduction

Iron (Fe) is an essential element for all living organisms because it is a cofactor for fundamental biochemical activities, such as

energy metabolism, oxygen transport and DNA synthesis. Because of its redox reactivity [Fe shuttles between the reduced ferrous (Fe^{2+}) and the oxidised ferric (Fe^{3+}) forms], which allows it to associate with proteins and bind to oxygen, Fe transfers electrons and mediates catalytic reactions (Aisen et al., 2001). Fe is also potentially toxic because it can catalyse the propagation of reactive oxygen species (ROS) and the generation of highly reactive radicals (such as the hydroxyl radical) through Fenton chemistry (Koppenol, 1993). The ensuing oxidative stress is associated with damage to cellular macromolecules, tissue injury and

Abbreviations: mETC, mitochondrial electron transport chain; ND_{in} , internal type II NADPH dehydrogenases; ND_{ex} , external type II NADPH dehydrogenases.

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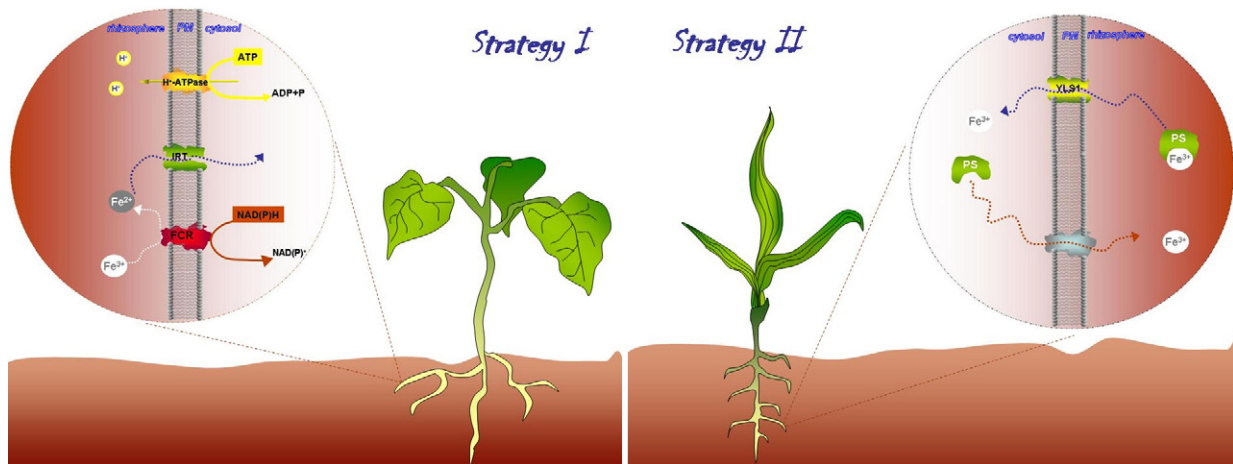


Fig. 1. Schematic representation of the mechanisms of iron uptake in plant roots. Left panel: Dicotyledonous plants induce the so-called Strategy I mechanism, characterised by the induction of the FCR, IRT1 and H⁺-ATPase at the plasma membrane level. Right panel: monocotyledonous plants induce the so-called Strategy II mechanism, characterised by the production and the extrusion of the PS in the rhizosphere, and the uptake of the Fe³⁺-PS complexes by the plants. *Abbreviations:* FC-R, ferric chelate-reductase; IRT1, iron-regulated transporter; PM, plasma membrane; PSs, phytosiderophores; YSL1, yellow strip-like 1.

disease (Galaris and Pantopoulos, 2008; Kell, 2009). Notably, the bioavailability of oxidised Fe³⁺ is poor due to the limited solubility of its compounds. Thus, the acquisition, usage and detoxification of Fe poses a considerable challenge for cells and organisms, which have evolved sophisticated mechanisms to satisfy their metabolic needs and, concomitantly, minimise the risk of toxicity (Andrews, 2008; De Domenico et al., 2008; Hentze et al., 2010). In plants, most of the concern about Fe nutrition is related to its low availability in soil solutions. In fact, notwithstanding its abundance, Fe exists in well-aerated soils as scarcely soluble oxides and oxyhydroxides and is therefore not freely available for plant uptake. To cope with this problem, plants have developed two main strategies: graminaceous plants use a chelation-based strategy (Strategy II), whereas the majority of plants, dicotyledons and non-graminaceous monocotyledons, use a reduction-based strategy (Strategy I) (Schmidt, 1999; Curie and Briat, 2003; Abadía et al., 2011) (Fig. 1). The chelation-based mechanism (Strategy II) has evolved in grasses, which includes most of the world's staple grain crops. Grasses produce molecules of the mugineic acid family called phytosiderophores (PSs). The PSs form a stable, hexadentate complex with Fe³⁺, which is the predominant form of Fe found in aerobic soils. PSs are secreted into the rhizosphere where they chelate and help to solubilise Fe³⁺. The Fe (III)-PS complex is then absorbed by the root cells through the action of Yellow Stripe1 (YS1) proteins (von Wiren et al., 1999; Curie et al., 2001; Abadía et al., 2011) (Fig. 1, right panel). In Strategy I, Fe acquisition is mediated by a reduction-based mechanism: a ferric chelate reductase (FCR) converts Fe (III)-chelates to Fe (II), and an iron regulated transporter (IRT1) moves the ion across the plasma membrane (PM) into the cell. Additionally, in most of the Strategy I plants studied to date, there is an associated increase in the activity at the PM, i.e., H⁺-ATPase transports ions across the membrane, which lowers the pH of the growth medium, increases the Fe solubility and generates the electrochemical proton gradient necessary to drive ion uptake (Rabotti and Zocchi, 1994; Dell'Orto et al., 2000; Santi et al., 2005; Santi and Schmidt, 2008, 2009) (Fig. 1, left panel). The genes encoding these enzymes have been identified in several plants (Eide et al., 1996; Robinson et al., 1999; Eckhardt et al., 2001; Waters et al., 2002, 2007; Santi et al., 2005). Additionally, in Strategy I plants a plethora of organic compounds, including carboxylates, phenolics, and flavonoids, are increased under Fe deficiency (Cesco et al., 2010; Tomasi et al., 2008).

Under Fe-limited growth conditions, the induction of the Strategy I mechanism is not restricted to the activation of FCR, IRT1

and H⁺-ATPase. A metabolic reprogramming of plant root cells is necessary; the activation of reduction processes and the enhanced proton extrusion require an adequate energy supply in the form of NADPH and ATP. The recharging of such substrates requires the acceleration of the metabolism that is strictly linked to energy production (Zocchi, 2006; Vigani and Zocchi, 2009). In fact, several papers published in the last 30 years revealed that almost all enzymes belonging to the glucose catabolism, mainly at the glycolysis level, are increased in several Strategy I plants (Table 1). Among the metabolic activities that increase in Fe-deficient plants, phosphoenolpyruvate carboxylase (PEPC) is one of the most important (see references in Table 1). PEPC is a ubiquitous enzyme in plants that catalyses the fixation of bicarbonate to phosphoenolpyruvate (PEP) to produce oxaloacetate (OAA) and Pi. To maintain an

Table 1

Experimental evidences about the increase of some cytosolic enzymes of glucose catabolism under Fe deficiency in several Strategy I plants. References are tabulated according to the type of induction (activity and/or protein content and/or transcript) observed under Fe deficiency with regard the specific enzyme. The numbering of references is specified below.

Enzyme	Activity	Protein	Transcript
HK	7		
PGI			
ATP-PFK	5; 7; 11; 12	14; 15	
PP-PFK		14	
F1,6BP		8; 13; 14; 15	6
G3PDH	1; 2; 5; 11; 12	12; 13; 15	6
TPI		8; 13	6
PGK		13; 14	6
PGM		8; 14; 15	6
Enolase		8; 13; 14; 15	6
PK	5; 7; 11; 12		6
PEPC	2; 3; 4; 7; 9; 10; 11; 12; 16	3; 8; 16	6; 10; 17
G6PDH	1; 2; 4; 11; 12	14; 15	

(1) Sijmons and Bienfait (1983); (2) Rabotti et al. (1995); (3) De Nisi and Zocchi (2000); (4) López-Millán et al. (2000a,b); (5) Espen et al. (2000); (6) Thimm et al. (2001); (7) Zocchi et al. (2007); (8) Li et al. (2008); (9) López-Millán et al. (2009); (10) Andaluz et al. (2009); (11) M'Sehli et al. (2008); (12) Jelali et al. (2010); (13) Rellán-Álvarez et al. (2010); (14) Donnini et al. (2010); (15) Rodríguez-Celma et al. (2011); (16) Slatni et al. (2011); (17) De Nisi et al. (2010). *Abbreviations:* ATP-PFK, ATP-dependent phosphofructokinase; F1,6BP, fructose 1,6-bisphosphate aldolase; G6PDH, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HK, hexokinase; PEPC, phosphoenolpyruvate carboxylase; PGI, phosphoglucosomerase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; PP-PFK, PP-dependent phosphofructokinase; TPI, triosephosphate isomerase.

adequate rate of glycolysis, PEP must be consumed at a high rate. Under conditions of Fe deficiency, it has been postulated that the activation of PEPC could be the downstream driving force for glycolysis that leads to an increase in the rate and production of cytosolic acidification to activate H⁺-ATPase (Zocchi, 2006). Additionally, the activation of PEPC could be responsible for the production of organic acids. Indeed, when under Fe deficiency, plants produce considerable amounts of organic acids, mainly citrate and malate (Abadía et al., 2002 and references therein). The following is a list of potential role(s) of increased organic acids under Fe deficiency: (i) to control the cytosolic pH or feed the increased activity of H⁺-ATPase at the PM; (ii) to increase production of oxalacetate (as a product of PEPC) and/or malate to replenish the tricarboxylic acid (TCA) cycle; and/or (iii) to facilitate the acquisition of Fe from the soil by the root cell (Abadía et al., 2002).

Overall, the upregulation of glycolysis under Fe deficiency provides: (i) ATP synthesis to sustain the major activity of the H⁺-ATPase; (ii) the formation of reducing equivalents for the FC-R; (iii) the formation of PEP and (iv) a contribution to the regulation of cytosolic pH. Because Fe deficiency induces an energy demand in the cell, it is critical to understand how this stress affects mitochondria.

Plant mitochondria and iron

Mitochondria play a key role in energy transduction, and are the site of aerobic respiration, a process common to almost all eukaryotic organisms that releases a large amount of free energy in the cell. Mitochondria possess two membranes that divide the organelle into four compartments: (i) the outer membrane (OMM); (ii) the region between the two membranes or intermembrane space (IMS); (iii) a highly invaginated inner membrane (IMM); and (iv) the matrix, which is the aqueous phase contained within the inner membrane (Sweetlove et al., 2007). The IMM is the site of oxidative phosphorylation (OXPHOS) electron transport chain coupling NADH and FADH₂ oxidation, and reduction of oxygen, to proton translocation and OXPHOS. The TCA cycle, which is responsible for the oxidative decarboxylation of organic acids leading to reduction of NADP and FAD and substrate-level phosphorylation of ADP to ATP, takes place in the matrix (Millar et al., 2011 and references therein).

The mitochondrion occupies a central place in the metabolic network of eukaryotes. Essential metabolic processes occur within the organelle, and several other metabolic pathways either converge on or derive from the mitochondrion. In plants, mitochondria provide precursors for a number of essential biosynthetic processes (e.g., nitrogen fixation and the biosynthesis of amino acids, tetrapyrroles and vitamin cofactors, Douce, 1985; Douce and Neuberger, 1989; Mackenzie and McIntosh, 1999).

Interestingly, mitochondria are also a focal point of Fe metabolism. An analysis of the metalloome of mitochondria isolated from *Arabidopsis* revealed a 26:8:6:1 molar ratio for iron:zinc:copper:manganese (Tan et al., 2010), suggesting that Fe is the primary micronutrient present in the mitochondrion (Nouet et al., 2011). Mitochondria contain a large amount of metalloproteins that require Fe to carry out their function (Bertini and Rosato, 2007). In fact, several enzymes belonging to both the respiratory chain and to the TCA cycle are Fe-containing proteins. Moreover, crucial steps of the Fe–S cluster assembly for the entire cell take place in the mitochondria, suggesting an important role for this compartment in Fe handling by the cell.

Iron deficiency and mitochondria are strongly linked because this stress greatly induces the Strategy I activities that lead to a great energy demand, thus the participation of the mitochondria would be required.

Iron uptake and handling in plant mitochondria

Once inside the root cell, Fe can face different fates: (i) chelation by citrate and stored in vacuole or transported to the shoot; (ii) sequestration by ferritin in the cytosol; or (iii) transportation to organelles, such as mitochondria (Conte and Walker, 2011). The processes of Fe transport into plant mitochondria are partially unknown. The OMM permeability is conferred by a family of porin proteins, forming channels for the free diffusion of a wide range of molecules, and no specific Fe transporter has been identified so far. In the IMM, the Fe importer MIT1 (Mitochondrial Iron Transporter 1) has been recently identified, which is essential for plant growth and development (Bashir et al., 2011). Furthermore, the ABC transporter STA1/AtATM3 (ATP-binding cassette Transporters of Mitochondria 3) has been characterised in *Arabidopsis* and implicated in the export of Fe–S clusters (Kushnir et al., 2001). In addition, FCR(s), encoded by *FRO* genes in plants, might be involved in mitochondrial Fe transport because AtFRO3 and AtFRO8 contain mitochondria-targeting sequences and are mainly located in root and shoot veins, respectively (Mukherjee et al., 2006). These findings suggest that a reduction-based uptake could also take place in the IMM of mitochondria. Moreover, the oxidising conditions found in the mitochondrial IMS (Hu et al., 2008) would indicate the need for a reduction-based mechanism (Abadía et al., 2011).

Once Fe is inside the mitochondria, it can be utilised in several pathways. The most important pathways for mitochondrial functionality and cell viability are Fe–S cluster assembly, haem biosynthesis and Fe storage.

Iron–sulphur (Fe–S) clusters are cofactors that are chemically simple but functionally versatile. In fact, they represent a prosthetic group of proteins that are essential to life processes, such as photosynthesis, respiration and nitrogen fixation. Fe–S clusters consist of Fe and acid-labile sulphide (Balk and Lobréaux, 2005). Plant mitochondria operate the so-called ISC (Iron Sulphur Cluster) pathway for the assembly of the Fe–S cluster. Recently, this pathway was extensively reviewed (Balk and Pilon, 2011). Interestingly, the Fe–S cluster assembly capability of plant mitochondria is essential not only for mitochondrial Fe–S proteins but also for cytosolic Fe–S protein synthesis. Indeed, in the cytosol, the so-called Cytosolic Iron–sulphur protein Assembly (CIA) machinery is present. Cytosolic Fe–S protein synthesis depends on ISC components and a mitochondrial ABC transporter (ATM3) that has been proposed to export an unidentified product of the ISC pathway, allowing Fe–S cluster assembly and therefore Fe–S protein synthesis in the cytosol by the CIA machinery (Balk and Pilon, 2011 and references therein).

There is also evidence that mitochondria can synthesise haem. Haem is a prosthetic group formed by an Fe atom that coordinates the binding of a large heterocyclic organic ring called a porphyrin. Not all porphyrins contain Fe, but a substantial fraction of porphyrin-containing metalloproteins has haem as their prosthetic group: the so-called haemoproteins. Haem forms the prosthetic group of photosynthetic and respiratory cytochromes involved in energy transduction and oxidases, such as catalase, peroxidase and NADPH oxidase (Sweetlove et al., 2007 and references therein). In plant cells, the presence of the haem biosynthetic pathway in plant mitochondria is still a matter of debate. However, some authors report that the enzymes that catalyse the final steps of haem biosynthesis, such as ferrochelatase (which catalyses the insertion of ferrous Fe into protoporphyrin IX) and protoporphyrinogen oxidase, are also present in mitochondria and in plastids (Sweetlove et al., 2007 and references therein).

The mitochondrial proteins that are critical for Fe homeostasis are ferritin and frataxin. Generally, ferritin is considered the primary Fe storage protein in the cell. Ferritin provides protection against Fe toxicity and oxidative damage, and its expression is

increased upon exposure to an excess of Fe (Eisenstein, 2000; Arosio et al., 2009; Briat et al., 2010a,b). Zancani et al. (2004) reported the mitochondrial localisation of ferritin in both *Pisum sativus* and *Arabidopsis thaliana*. Moreover, recently, the ferritin isoform FER4 was identified in the mitochondria in *Arabidopsis* heterotrophic cells (Tarantino et al., 2010b). A *fer4 Arabidopsis* mutant displays different rearrangements of its respiratory machinery but is not impaired in its development or response to abiotic stress (Tarantino et al., 2010a,b). Although the precise role of ferritin in plant mitochondria has not been resolved completely, there is evidence for its role in Fe homeostasis. Frataxin is a conserved mitochondrial protein implicated in cellular Fe homeostasis, which functions as an Fe chaperone that delivers Fe for the Fe–S cluster and haem biosynthesis (Malinardi et al., 2011 and references therein). Frataxin deficiency causes severe disease in both humans and plants; the absence of frataxin induces severe oxidative stress in mitochondria, while its overexpression allows the cell to be more resistant to oxidising agents (Møller et al., 2011 and references therein).

Overall, it is increasingly evident that mitochondria can be considered as an 'iron-handling area' within the cell, possessing several specific enzymes for the maintenance of this element. Mitochondria store and process Fe to synthesise a large number of essential proteins for their own functionality and for that of the entire cell.

Mitochondrial respiration and Fe deficiency

In the mitochondria of all eukaryotes, the principal respiratory electron transport chain consists of a series of membrane-bound redox centres that catalyse the multi-step transfer of electrons from NADH and FADH₂ to oxygen. The mitochondrial electron transfer chain (mETC) facilitates a number of exergonic redox reactions and conserves energy through proton translocation. The standard mETC consists of four multi-centred protein complexes (referred to as Complexes I through IV). In addition, the F₁F₀-ATP synthase (Complex V), which does not have electron transfer activity, provides a path for passive proton diffusion into the matrix and uses the free energy released by this spontaneous movement to drive the phosphorylation of ADP. In addition to the electron transfer chain shared by all aerobic eukaryotes, plant mitochondria also possess novel pathways for both the oxidation of NADH and the reduction of oxygen. These bypass proteins, the so-called alternative type II NADPH dehydrogenases [located both in the external surface of the IMM (ND_{ex}) and the internal surface of the IMM (ND_{in})] and the alternative oxidase (AOX), do not pump protons, and the free energy released as electrons flow through them is lost as heat. These additional enzymes allow a great deal of flexibility in the oxidation of NADPH in plants. Type II NADPH dehydrogenases reduce ubiquinone and thus circumvent respiratory Complex I, whereas AOX bypasses Complexes III and IV of the cytochrome pathway by directly oxidising ubiquinol. Millar et al. (2011) recently reviewed the specific molecular and genetic features of each enzyme of the respiratory chain.

In the absence of Fe, the levels of some Fe-containing components (cytochromes and Fe–S clusters) were greatly diminished in the mitochondria of Fe-deficient sycamore cells (Pascal and Douce, 1993) and cucumber roots (Vigani et al., 2009). In Fe-deficient sycamore cells, the activity of Complex II was affected, whereas Complexes I, III, and IV, and ATP synthase, were unimpaired. In contrast, in cucumber root, the number of Fe ions required for the correct assembly of each mETC complex (unit) and their relative activities under Fe-deficient conditions are highly correlated: Complex IV, which has only two Fe atoms, showed a decrease in activity of approximately 50%, whereas Complexes I and II, which require at least 20 and 10 Fe atoms, respectively, showed a greater decrease (95% and 77%, respectively). A proteomic

investigation of *Medicago truncatula* roots under Fe deficiency showed a decreased level of some Fe-containing proteins related to the respiratory chain (e.g., 76 kDa mitochondrial Complex I) (Rodríguez-Celma et al., 2011). In contrast, a proteomic study performed on Fe-deficient *Arabidopsis* roots showed an increased level of several proteins belonging to the respiratory chain. These proteins included cytochrome c oxidase, the ubiquinol-cytochrome c reductase complex ubiquinone-binding protein and three components of mitochondrial Complex I (Lan et al., 2011). The contrasting results obtained in cucumber compared with *Arabidopsis* might be explained by the different degree of Fe deficiency conditions used in the studies.

Overall, root mitochondria are strongly affected by Fe starvation because they require at least forty 40 Fe atoms per respiratory unit to function (Vigani et al., 2009). Nevertheless, when Fe availability is low, either due to absence, as in the cucumber study, or low supply (Fe content in the nutrient solution is low but not zero), as in the *Arabidopsis* (Lan et al., 2011) and sycamore cell (Pascal and Douce, 1993) studies, the mitochondria are still working.

Under increased Fe deficiency, it is possible to observe an interesting regulation of the respiratory chain in cucumber root; the dehydrogenase pathways (the enzymes able to reduce the UQ pool) of the mETC are more affected with respect to the cytochrome pathways (the enzymes able to oxidase the UQ pool). The strong decrease of Complex I and Complex II activities in the absence of Fe is compensated by the activation of the type II alternative NADPH dehydrogenase (see above). In fact, both the enzymatic activity and the protein level of the ND_{ex} are highly induced compared with the ND_{in} (Vigani and Zocchi, 2010). These findings suggest that the respiratory chain could contribute to cytosolic NADPH turnover through the ND_{ex}, notwithstanding that the mETC is strongly inhibited by Fe deficiency (Vigani and Zocchi, 2010). Accordingly, Higa et al. (2010) observed an induction of type II alternative NADPH dehydrogenase in the Fe-deficient roots of *Hyoscyamus albus* that was similar to that in cucumber, where low Fe availability decreases the main dehydrogenase enzymes of the mETC (Complexes I and II) and induces the alternative dehydrogenase pathways. Interestingly, Higa and co-workers (2010) stated that Complex I and Complex II require not only a large number of Fe ions but also flavins as cofactors. Interestingly, flavin accumulation and excretion are induced upon Fe deprivation in various plant species (Pound and Welkie, 1958; Nagarajah and Ulrich, 1966; Kannan, 1988; Welkie et al., 1990). It has been suggested that flavins, which accumulate in Fe-deficient roots, could act as electron donors or as cofactors for the FCR (Rodríguez-Celma et al., 2011; López-Millán et al., 2000a,b; Gonzalez-Vallejo et al., 1998; Welkie and Miller, 1993) because this enzyme contains an FAD-binding sequence motif (Schagerlof et al., 2006). However, it is rational to consider that if Complex I and Complex II proteins were prevented from becoming fully functional by Fe deficiency, the unused flavins might be transported outside the mitochondria, reaching the apoplast and the rhizosphere (Higa et al., 2010).

Generally, Fe deficiency affects the respiratory chain by limiting the activity of Fe-containing enzymes and inducing the alternative enzymes. This regulation allows the respiratory chain to partially satisfy the energy demand of the cell.

TCA cycle and Fe deficiency

The TCA cycle is a universal feature of the metabolism in aerobic organisms. It begins with the condensation of OAA and acetyl-CoA, proceeds via a series of oxidative steps that release two carbon atoms as CO₂, and ends with the regeneration of OAA (Fig. 2). In plants, acetyl CoA is derived from the end product of glycolysis, pyruvate, by the action of mitochondrial pyruvate dehydrogenase.

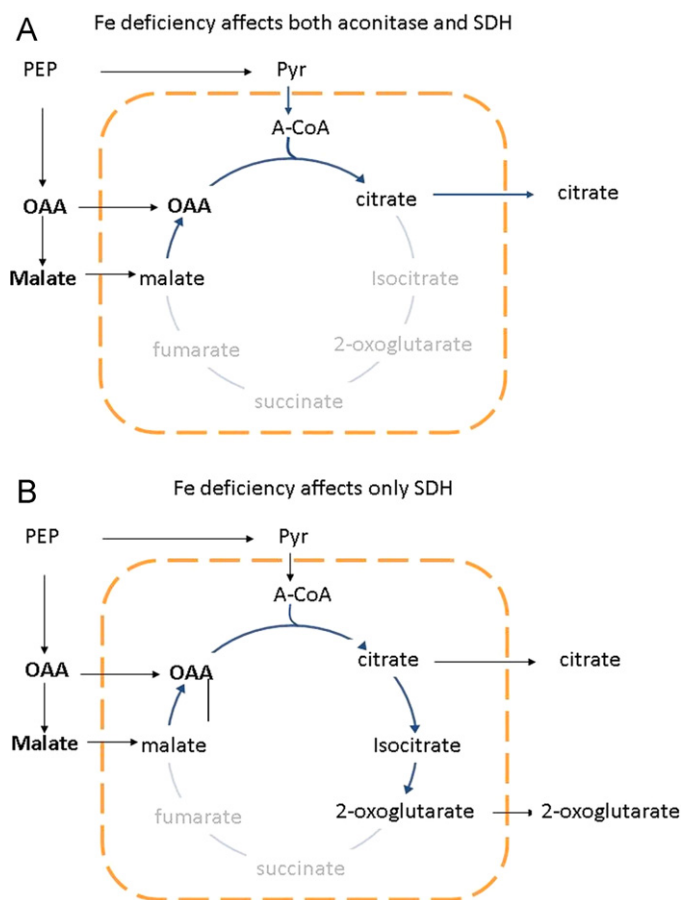


Fig. 2. Representation of the changes occurring at the TCA cycle level under Fe deficiency. (A) Hypothetical non-cyclic TCA mode occurring in the case of decrease of aconitase and SDH activities under Fe deficiency. (B) Hypothetical non-cyclic TCA mode occurring in the case of decrease of SDH activity under Fe deficiency.

The pyruvate is either imported directly from the cytosol or synthesised by the mitochondrial NAD-malic enzyme from malate that, in turn, is derived from cytosolic PEP. PEP carboxylase generates cytosolic OAA, which is readily converted to malate and aspartate. All three metabolites can be imported into the mitochondria to provide a source of malate for the NAD-malic enzyme (Sweetlove et al., 2010).

Several authors have suggested that Fe deficiency induces the activation of the TCA cycle. Li et al. (2008) observed that the expression of several TCA enzymes (isocitrate dehydrogenase, aconitase, succinyl-CoA ligase, and succinate dehydrogenase) was simultaneously upregulated in Fe-deficient tomato roots. López-Millán et al. (2009) showed that the total organic anion concentrations in tomato root tips did not change significantly with Fe deficiency, but their composition was markedly changed. In the control root tips, the major organic anions were oxalate, 2-OG and malate, whereas in Fe-deficient root tips, citrate and malate accounted for 90% of the total organic anion concentrations. Moreover, the authors showed that the activities of five enzymes (ICDH, aconitase, MDH, fumarase and CS) involved in organic acid metabolism increased significantly in the tissue extracts of Fe-deficient root tips when compared with the Fe-sufficient tips. The authors pointed out that the upregulation of the TCA cycle might have special significance for roots responding to Fe deficiency. The TCA cycle provides abundant organic acids, including citrate and malate, which play an important role as chelators for Fe transport in plants (López-Millán et al., 2000b; Tiffin, 1966). A metabolite study in *Beta vulgaris* revealed large increases in organic acids related to the TCA cycle (Rellán-Álvarez et al., 2010).

Additionally, a proteomic analysis performed on *Medicago truncatula* roots revealed an increase in the amount of several TCA cycle enzymes under Fe-deficient conditions (Rodríguez-Celma et al., 2011). All these data suggest that the TCA cycle plays an important role in the Fe deficiency response by providing organic acids and protons for the acidification of the rhizosphere.

Nevertheless, none of the studies mentioned above involved the isolation of purified mitochondria. Therefore, they were unable to discriminate between the identical reactions occurring in the mitochondrial matrix and in the cytosol (i.e., reactions catalysed by aconitase, malate dehydrogenase and isocitrate dehydrogenase). One complication inherent in studying the TCA cycle is that almost all the steps in the pathway can be bypassed by steps in the cytosol; however, the reactions catalysed by citrate synthase and succinate dehydrogenase are exceptions.

Like the respiratory chain, the TCA cycle possesses two Fe-containing enzymes [i.e., aconitase and the succinate dehydrogenase (Complex II of the respiratory chain)]. As previously discussed, the succinate dehydrogenase activity in purified mitochondria obtained from cucumber roots (Vigani et al., 2009) and sycamore cells (Pascal and Douce, 1993) strongly decreased under Fe deficiency, suggesting that the TCA cycle was decelerated at least at some point.

Sweetlove et al. (2010) recently reviewed the different carboxylic acid metabolism flux modes predicted from metabolic models and concluded that cyclic TCA could not be just a cycle. They propose that the cyclic mode of TCA provides efficient energy transfer from the carbon bonds of respiratory substrates to ATP, suggesting that it might not be necessary to maintain the cyclic flux if the demand for ATP is low or if alternative sources of ATP are available. Consistent with this argument, a recent flux-balance model of heterotrophic *Arabidopsis* metabolism demonstrates that a cyclic TCA flux is only established as the demand for ATP increases in a cell (Poolman et al., 2009; Sweetlove et al., 2010).

It was proposed that the effect of Fe deficiency on the TCA cycle could induce different changes. Considering the expected decrease of aconitase and succinate dehydrogenase activities under Fe deficiency, the TCA cycle could shift from a circular to a linear mode, creating at least two metabolic scenarios (Fig. 2). First, if both aconitase and succinate dehydrogenase activities are strongly affected by Fe deficiency, the TCA cycle could similarly work as in the non-cyclic flux mode as predicted from the analysis of an enzyme kinetic model (Steuer et al., 2007); malate is imported into the matrix and then converted to citrate, which is subsequently exported from mitochondria (Fig. 2A). This hypothetical scenario is plausible because there is evidence for the accumulation of the di- and tricarboxylic acid carrier (DTC) protein under Fe deficiency, suggesting an increased transfer of organic acids, mainly malate and citrate, between the mitochondria and cytosol. Additionally, increased citrate export from the mitochondria to the cytosol has been observed in several species under Fe deficiency (Vigani and Zocchi, 2009 and references therein). However, there is no direct evidence that mitochondrial aconitase is affected by Fe deficiency, even if it would be expected.

Indeed, the second scenario implies that Fe deficiency affects only succinate dehydrogenase. In this case, the TCA cycle could work as in the non-cyclic flux mode predicted under conditions of low ATP demand in a genome-scale metabolic model of *Arabidopsis* (Poolman et al., 2009; Sweetlove et al., 2010); malate and/or OAA are imported into the matrix and then converted to 2-OG, which is subsequently exported from the mitochondrion together with citrate (Fig. 2B). This hypothesis is also reasonable because there is evidence for the strong inactivation of Complex II under Fe deficiency (Pascal and Douce, 1993; Vigani et al., 2009).

Taking into consideration that the TCA cycle does not necessarily support a cyclic flux and that the actual flux distribution

through it will reflect the function of the whole metabolic network, a combination of these non-cyclic TCA modes could occur under Fe deficiency. Furthermore, the presence of a cyclic or non-cyclic mode of TCA might depend on the degree of stress. Indeed, it could be hypothesised that when the amount of Fe in the cell is close to zero, Fe-containing enzyme activities are inhibited, which leads to a non-cyclic TCA mode. When the amount of Fe is low but not depleted, the Fe-containing enzymes might still function, leading only to a slowing of the TCA cycle. In any case, the shift from a cyclic to a non-cyclic TCA mode under Fe deficiency could result from a necessity to maintain a functioning metabolism, even if important Fe-containing enzymes are not synthesised.

Mitochondrial dynamics in Fe-deficient plants

The morphology and ultrastructure of the mitochondria are also affected by Fe deficiency. In fact, mitochondria from Fe-deficient sycamore cells displayed a more dilute matrix with less pronounced cristae (Pascal and Douce, 1993). Interestingly, in Fe-deficient cucumber roots, mitochondria displayed a particular handlebar-like structure and appeared to aggregate (Vigani et al., 2009) (Fig. 3A). However, the particular shape that was observed in Fe-deficient cucumber roots was also observed in the wild-type heterotrophic cells of *Arabidopsis* grown under control conditions, indicating that this shape is probably not specifically related to Fe deficiency (Tarantino et al., 2010b). A similar handlebar-like structure observed in Fe-deficient cucumber root has been observed in the *Arabidopsis drp3a* mutant (Logan, 2006, 2010) (Fig. 3). DRP3A is a dynamin-like protein that is required for the maintenance of mitochondrial size and number in *Arabidopsis* and rice; this protein has been partially localised to the poles of mitochondria and to sites of constriction (Arimura et al., 2004; Fujimoto et al., 2004; Logan et al., 2004). The disruption of DRP3A results in an aberrant mitochondrial morphology that is characterised by an increase in the size of individual mitochondria and a corresponding decrease in the number of mitochondria per cell (Logan, 2010 and references therein). Interestingly, *drp3a* mutants displayed mitochondria with the presence of a matrixule, a thin protuberance of up to many micrometers in length that extends from one pole of a mitochondrion (Fig. 3B). It has been proposed that the presence of numerous mitochondria with matrixules in *drp3a* mutants is linked to the function of DRP3A as a component of the mitochondrial division machinery (Logan, 2010 and references therein).

The higher plant chondriome (all the mitochondria in a cell, collectively) is a highly dynamic structure composed predominantly of physically discrete organelles. This structure contrasts with that of most animal cell types and yeast cells in which the chondriome is frequently organised into long tubules or reticula. An analysis of the mitochondrial morphology *in vivo* has shown that higher plant mitochondria are highly pleomorphic, although most frequently they are spherical to sausage-shaped organelles. Mitochondria are also dynamic in terms of their movement within cells, moving rapidly along cellular structures, such as actin and microtubule cytoskeletal elements (Logan, 2006, 2010 and references therein). The primary mitochondrial processes that regulate the shape and the movement of the organelles in the cell are fusion and fission. These two processes compensate for one another, leading to the maintenance of a relatively constant number of mitochondria in the cell (Logan, 2006, 2010). Several authors have reported that the number of mitochondria was increased in Fe-deficient cells (Landsberg, 1986, 1994; Pascal and Douce, 1993; Dell'Orto et al., 2002); therefore, it is rational to hypothesise that this nutritional stress could modify the mitochondrial fusion/fission ratio.

Because the plant chondriome is organised as a discontinuous whole, proper mitochondrial function requires fine control over

the mitochondrial motility and cellular distribution. In fact, it is intrinsic in the discontinuous whole hypothesis that there is a 'need to meet', where the need is to provide a mechanism for the exchange of mitochondrial proteins; the fusion of physically discrete organelles requires mitochondrial movement (Logan, 2010). The control of mitochondrial positioning and direction of movement are the result of the coordinated activity of myosin and the rate of actin turnover (Logan, 2010).

Under Fe deficiency, the amount of actin decreases (Donnini et al., 2010), whereas the number of mitochondria increases. Mitochondrial motility has not been studied in Fe-deficient plants, and we do not know exactly how cells organise the cytoskeleton to allow organelle and vesicle trafficking in the cytosol. We might speculate that the increased number of mitochondria observed in Fe-deficient roots in several studies could be in response to an inefficient cytoskeletal structure to enhance the probability of mitochondrial contact, thus maintaining the discontinuous whole structure of the plant chondriome. In this way, the cell could satisfy the "need to meet" hypothesis. Interestingly, under Fe deficiency, mitochondrial contact results from a strongly affected metabolism in which the merging of mitochondria could help them to overcome the lack of Fe and at the same time, facilitate cell survival. Moreover, the probable shift towards a non-cyclic TCA mode increases mitochondrial communication with external components, within the cytosol and with other mitochondria. The fact that under a mild Fe deficiency, one of the first enzymes affected is Complex II (Pascal and Douce, 1993) would mean that mitochondria, perceiving an upcoming Fe deficiency, reprogram their own function, adjusting both the respiratory chain and the TCA cycle by acting on only one enzyme.

Role of mitochondria in controlling the NADPH reduction levels under Fe deficiency

The presence of type II NADPH dehydrogenases with different properties on both sides of the mitochondrial inner membrane allows the mETC to specifically regulate the reduction of either mitochondrial or cytosolic NADH or NADPH (Rasmusson and Wallström, 2010 and references therein). Importantly, metabolite exchangers, together with the activity of the TCA cycle enzymes, mediate reductant shuttling across the inner mitochondrial membrane, modulating the turnover of reducing equivalents in both the mitochondria and the cytosol. Indeed, the reduction of NADH in the matrix and the cytosol are believed to equilibrate primarily with the malate/OAA ratios in each compartment, while the reduction of NADPH pools between the matrix and cytosol is equilibrated through the export of reductant from mitochondrion to the cytosol by both a citrate valve that exchanges citrate and 2-oxoglutarate (Igamberdiev and Gardeström, 2003) and a malate valve that exchanges malate and OAA (Scheibe, 2004).

In this context, the reducing power turnover of Fe-deficient cells undergoes a fine and complex regulation to satisfy the metabolic request. Indeed, because both the cytosolic NADPH and the mitochondrial NADPH need to be re-oxidised to keep the catabolic pathways working, a less active respiratory chain could impair this turnover, leading to a higher reduced/oxidised NADP ratio, as shown by the increased NADPH/NADP⁺ ratio in Fe-deficient bean and *Plantago lanceolate* (Sijmons et al., 1984; Schmidt and Schuck, 1996). However, in Fe-deficient sugar beet and tomato roots, the NADPH/NADP⁺ ratio was lower than the relative control conditions (López-Millán et al., 2000a,b, 2009), suggesting that Fe deficiency might stimulate the NADPH-oxidising activities more than the NADP⁺-reducing activities.

To oxidise the large production of NADPH from metabolism, the cell activates reactions that are able to consume NADPH.

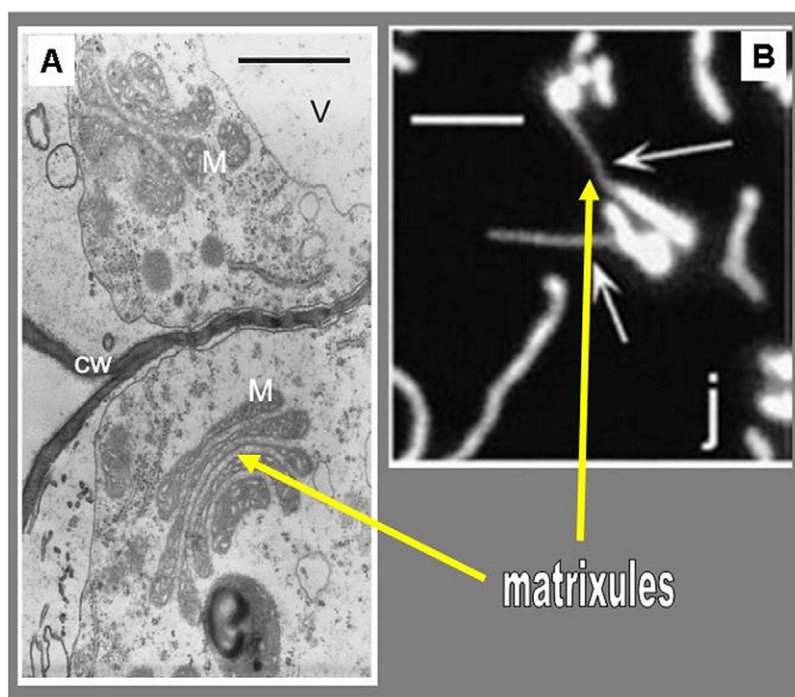


Fig. 3. Morphological comparison of mitochondria from the Fe-deficient cucumber root (Vigani et al., 2009; kind permission to reproduce from John Wiley and Sons, license number: 2740690508918) and *dpr3a* mutant of *Arabidopsis* (Logan et al., 2004; kind permission to reproduce from Oxford University Press, license number: 2740640528797). (A) TEM analysis displaying the handlebar-like morphology observed in Fe-deficient cucumber roots (bar = 1 μm). (B) GFP imaging analysis of the mitochondria from *Arabidopsis dpr3a* mutants (bar = 10 μm). This mutant displayed mitochondria with the presence of matrixule, a thin protuberance of up to many micrometers in length that extends from one pole of a mitochondrion. Interestingly, similar matrixule formation seems to occur in Fe-deficient cucumber plants. *Abbreviations:* CW, cell wall; M, mitochondrion; V, vacuole.

López-Millán et al. (2000a,b, 2009) reported that Fe deficiency increased the activities of fermentative enzymes, such as lactate dehydrogenase (LDH), pyruvate decarboxylase (PDC) and several other cytosolic NADH-consuming enzymes, which could contribute to the oxidation of the pyridine nucleotide pool in tomato and sugar beet roots. These data were also confirmed by microarray analysis of Fe-deficient *Arabidopsis* (Thimm et al., 2001) and proteomic data obtained from cucumber roots, in which putative alcohol dehydrogenase and malate dehydrogenase proteins accumulated under Fe deficiency (Donnini et al., 2010). The mitochondria might participate in the oxidation of NADPH under Fe deficiency by the activation of their alternative pathways (i.e., NADPH DHs). The external and internal localisation of these enzymes in the inner mitochondrial membrane allows them to oxidise the NADPH on both the cytosol and the matrix sides (Vigani and Zocchi, 2010).

Because Fe deficiency leads to a strong energy demand in the cell, the activation of mitochondrial metabolism would represent the perfect solution for the cell. However, the lack of Fe strongly affects mitochondrial functionality. Nevertheless, the activity of the plant mitochondria results in a complicated reprogramming of their own metabolism. At the level of the respiratory chain, alternative pathways are induced, bypassing the impaired complexes. The TCA pathway could shift from a cyclic to a non-cyclic mode, bypassing the limiting step (for instance, succinate dehydrogenase) and improving external communication. This bypass might be accomplished by inducing both the anaplerotic reactions to produce organic acids (which are useful for the responses to Fe deficiency) and the metabolite shuttles to allow the recycling of reducing power. The cell, in some way, helps mitochondria satisfy their “need to meet”, which would be high under Fe deficiency, by increasing the number of mitochondria. To facilitate mitochondrial activity, the cell could obtain useful compounds for rhizosphere acidification and Fe chelation, such as organic acid and flavins, which result from the disuse of Complexes I and II (Fig. 4).

Overall, the metabolic alteration of Fe-deficient root cells depends on the mitochondrial functionality. This organelle, which represents the “Iron-Area” of a root cell, seems to be the main target of the Fe deficiency, which might also become a starting point for whole cellular metabolic reprogramming to overcome this emergency energetic status (Vigani and Zocchi, 2010). The Fe deficiency establishes a cellular effort in which mitochondria activate alternative pathways to sustain NADPH turnover and organic acid synthesis. In this way, mitochondria facilitate the increase of glycolysis, which becomes a strong source of energy substrates for the activation of the response to Fe deficiency.

Could mitochondria be involved in the signal transduction of the Fe-deficient status of the cell?

The mitochondrion occupies an integrated position in the metabolic network of plants, reflecting the evolutionary origins of the organelle. Indeed, through its integration into the ancestral eukaryotic cell, the mitochondrion provided a new arsenal of metabolic capabilities (Martin and Russell, 2003; Sweetlove et al., 2007). To communicate with external compartments, mitochondria generate several compounds as signals, such as ROS and organic acid. It is well known that mitochondria produce ROS that not only cause damage to cellular components but are also involved in intracellular signalling; H_2O_2 is considered the most likely messenger (Neil et al., 2002; Møller et al., 2011). Importantly, abiotic stress influences mitochondrial H_2O_2 production (Møller, 2001), which could generate secondary signals and therefore transduce the oxidative damage of the mitochondrial component to the cell (Halliwell, 2006; Rhoads et al., 2006). Indeed, because H_2O_2 does not have the required specificity to regulate specific genes, it has been proposed that (carbonylated) peptides deriving from the proteolytic degradation of oxidised protein could irreversibly be the specific secondary ROS messenger from

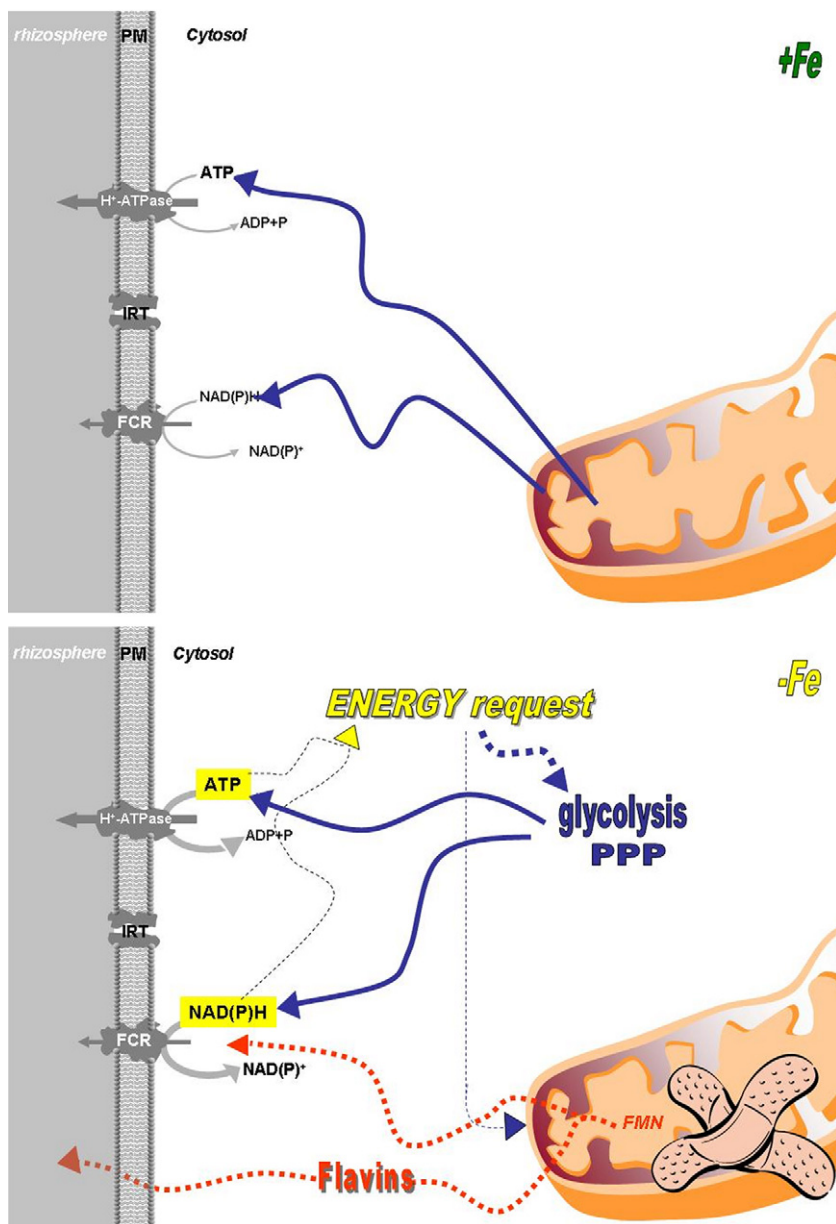


Fig. 4. Schematic representation of the role of mitochondria in the Fe-deficient dicotyledonous root cell. Under Fe deficiency, the activation of Strategy I responses (FCR, IRT1, H⁺-ATPase) requires a large supply of energetic substrates [i.e., ATP and NADPH]. Mitochondria cannot meet this energetic requirement because Fe deficiency affects several mitochondrial enzymes. Thus, the cell activates other pathways (e.g., glycolysis) to overcome the energetic emergency status. Nevertheless, mitochondria participate in metabolic responses induced by Fe deficiency by activating their own alternative respiratory pathways. Moreover, unused flavin compounds (i.e., FMN) could be transferred to the cytosol and extruded into the rhizosphere, facilitating the Fe reduction activity.

mitochondria to regulated source-specific genes (Møller and Sweetlove, 2010; Møller et al., 2011). The relationships between Fe deficiency and secondary oxidative stress remain controversial because iron is either a constituent or a cofactor of many antioxidant enzymes and simultaneously acts as a prooxidant through the Fenton reaction (Halliwell, 2006). However, Fe deficiency is associated with an accumulation of ROS (Donnini et al., 2011). In addition to ROS, it has been suggested that TCA-cycle intermediates might be potent regulators of gene expression in a manner analogous to sugars (Finkemeier and Sweetlove, 2006; Sweetlove et al., 2007). Indeed, the intermediates of the TCA cycle could communicate the mitochondrial metabolic status to the cytosol by virtue of the presence of di- and tri-carboxylic acid transporter (DTC) proteins located in the inner mitochondrial membrane (Picault et al., 2002). The exogenous application of citrate, malate and 2-oxoglutarate could induce AOX expression (Gray et al., 2004), suggesting that

it is the extra-mitochondrial levels of the TCA cycle metabolites that are sensed. Under Fe deficiency, a number of organic acids, such as citrate, are synthesised in the root cells from the TCA cycle. Moreover, DTC proteins accumulate in Fe-deficient cucumber roots (Vigani et al., 2009). The multiple roles for citrate are: Fe chelation, Fe transport, the regulation of cellular pH, and as a carbon skeleton source. Because of its multiple functions, citrate exported from the mitochondrion might be both in response to the Fe deficiency and an indicator of the metabolic status of the cell, together with the induction of several mitochondrial metabolic carriers that are able to strongly activate the exchange of organic acids and reducing equivalent compounds with the cytosol. Indeed, the cytosolic reducing equivalents might be transferred to the mitochondria through both specific reducing equivalent shuttles, such as glycerol-3-phosphate (G-3-P) shuttle (Shen et al., 2003, 2006) and several metabolite shuttles that operate between the two

compartments, including shuttles for malate/OAA (Ebbighausen et al., 1985) and malate/aspartate (Journet et al., 1981).

There is accumulating evidence for calcium (Ca^{2+}) involvement in mitochondrial signalling. In fact, Ca^{2+} itself might be an important part of the signal transduction pathways that originate from the mitochondria. Mitochondria contain relatively high concentrations of Ca^{2+} (Logan and Knight, 2003) and can be considered intracellular Ca^{2+} stores. Moreover, mitochondrial Ca^{2+} might be released into the cytosol during abiotic stress (Subbaiah et al., 1994), and there is evidence that mitochondrial Ca^{2+} levels respond to environmental signals (Logan and Knight, 2003). However, there is no clear evidence for Ca^{2+} involvement in the transduction of Fe deficiency-inducible responses. However, indirect findings suggest that Ca^{2+} might act as a second messenger in targeting the Fe deficiency signal in roots. In fact, Buckhout et al. (2009) reported the induction of genes encoding 14-3-3 proteins and some protein kinases in Fe-deficient *Arabidopsis* roots, which would be target proteins of the Ca^{2+} -calmodulin-transduced signal. Moreover, in Fe-deficient cucumber roots, it has been observed that a gene encoding calmodulin was overexpressed and its relative protein level was increased (Vigani et al., in press). Overall, these data suggest that Ca^{2+} is potentially involved in the transduction of Fe deficiency signals and that the mitochondria could participate in targeting the signal of altered Fe nutritional status.

Another well-known second messenger that could also be important in mitochondrial signal transduction is nitric oxide (NO). It is well documented that plants generate NO and that NO can act as an important signal molecule in a variety of scenarios (Wendehenne et al., 2004; Shapiro, 2005).

In plants, there are reports suggesting the existence of NO synthase (NOS)-like activities in various developmentally and physiologically stressful situations (Corpas et al., 2009). However, the existence of NOS in plant mitochondria is doubtful (Gupta et al., 2010). Indeed, it has been recently shown that plant mitochondria might be involved in NO turnover by the inter-conversion of nitrite and NO at the site of complex IV. Cytochrome *c* oxidase could represent the most plausible and established candidate for the nitrite-to-NO conversion, although other components of mETC could perform this reaction (Gupta and Igamberdiev, 2011 and references therein).

Importantly, NO has also recently been demonstrated to be involved in the induction of Fe-deficiency responses. Indeed, it has been shown that the NO level in roots was rapidly and continually elevated when plants were transferred to an Fe-deficient growth medium (Graziano and Lamattina, 2007; Jin et al., 2009; Chen et al., 2010). Furthermore, it has been observed that NO is required for the molecular events involved in controlling the development of lateral (Correa-Aragunde et al., 2004; Mendez-Bravo et al., 2010) and cluster roots (Wang et al., 2010), which are typical morphological changes that occur under Fe deficiency in several plants (Landsberg, 1994; Dell'Orto et al., 2002).

Thus, it is clear that mitochondria have several signal types that might be involved in Fe deficiency signal transduction.

Concluding remarks

As in other organisms, changes in the availability of Fe in plant mitochondria determine the reorganisation of cellular metabolism. The complex alteration of root metabolism under Fe deficiency has been reviewed here, discussing old and new findings. It is clear that mitochondria represent a central site of the metabolic regulation in the cell. In the case of low Fe availability, plant mitochondria become both a victim and a crucial site of fascinating alternative processes that make them unique in their role. The mitochondrion is probably the most affected compartment of the root cell under Fe

deficiency. Nevertheless, it is not destined just to succumb but to play a central role, through the effective modulation of the respiratory chain and the TCA cycle, in the Fe-deficient metabolic network. The mitochondrial flexibility in the development of the adaptive response to Fe deficiency is not only related to metabolism but also in mitochondrial dynamics. Moreover, the importance of mitochondria as possible signal-producing sites suggests an intriguing hypothesis that the mitochondrion might act as a cellular-stress sensor of Fe deficiency for the induction of the metabolic reprogramming of the cell.

Therefore, it would be of interest to concentrate future research on understanding how Fe deficiency influences mitochondria and *vice versa*, that is, how mitochondrial remodelling facilitates the reprogramming of root cell metabolism.

Acknowledgments

I wish to thank Prof. Graziano Zocchi and Dr. Marta Dell'Orto for critically reading the manuscript. I also wish to thank the anonymous reviewers for their precious comments to improve the manuscript.

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