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REVIEW

Nitric oxide and frataxin: two players contributing to maintain cellular iron homeostasis

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• *Background* Nitric oxide (NO) is a signalling and physiologically active molecule in animals, plants and bacteria. The specificity of the molecular mechanism(s) involved in transducing the NO signal within and between cells and tissues is still poorly understood. NO has been shown to be an emerging and potent signal molecule in plant growth, development and stress physiology. The NO donor *S*-nitrosoglutathion (GSNO) was shown to be a biologically active compound in plants and a candidate for NO storage and/or mobilization between plant tissues and cells. NO has been implicated as a central component in maintaining iron bioavailavility in plants.
• *Scope and Conclusions* Iron is an essential nutrient for almost all organisms. This review presents an overview of the functions of NO in iron metabolism in animals and discusses how NO production constitutes a key response in plant iron sensing and availability. In plants, NO drives downstream responses to both iron deficiency and iron overload. NO-mediated improvement of iron nutrition in plants growing under iron-deficient conditions represents a powerful tool to cope with soils displaying low iron availability. An interconversion between different redox forms based on the iron and NO status of the plant cells might be the core of a metabolic process driving plant iron homeostasis. Frataxin, a recently identified protein in plants, plays an important role in mitochondria biogenesis and in maintaining mitochondrial iron homeostasis. Evidence regarding the interaction between frataxin, NO and iron from analysis of frataxin knock-down *Arabidopsis thaliana* mutants is reviewed and discussed.

Key words: Nitric oxide, iron homeostasis, frataxin, strategy I, strategy II, dinitrosyl iron complexes, oxidative stress.

INTRODUCTION

Iron is an essential cofactor for a wide variety of cellular functions in all organisms. Iron is incorporated in the heme group of some proteins such as haemoglobin, myoglobin and cytochromes, or is associated with non-heme moieties or Fe–S motifs. It is required in essential processes for life such as oxygen transport, respiration, photosynthesis, hormone production and DNA synthesis (Briat and Lobréaux, 1997). Iron deprivation or difficulties in iron uptake or absorption cause anaemia in mammals and chlorosis in plants. In contrast, iron overload and pathologically increased iron uptake, surpassing the extracellular iron binding capacity as well as the intracellular iron storage capacity, lead to oxidant stress and permanent cell and tissue damage. Iron deficiency and overload can generate serious nutritional disorders, and as a result organisms have developed a tight regulatory control of iron homeostasis. Although impressive progress has been made in understanding the regulation points of iron homeostasis, many questions remain unanswered. Consequently, there is increasing interest in revealing the signal transduction pathways that control the perception, uptake, metabolism, storage and delivery of iron.

In mammals, adjustment of intracellular iron levels is mainly achieved by the coordinated regulation of the iron storage protein ferritin and the iron uptake protein system comprising transferrin (Tf) and its receptor (TfR1). Ferritin and TfR1 are regulated post-transcriptionally by particular hairpin structures called iron-responsive elements (IREs) contained in untranslated regions (UTRs) of mRNA (Richardson and Ponka, 1997). In iron deficiency, iron regulatory proteins (IRPs) bind to the ferritin mRNA IRE, which is found in the 5′-UTR, decreasing its translation. In parallel, IRPs also bind to the 3′-UTR TfR1 mRNA and increases its stability. As a result of these changes, iron uptake and availability increase within the cell. By contrast, high iron levels decrease IRE-binding activity, leading to efficient translation of ferritin and decreased stability of the TfR1 messenger, thus enhancing the sequestration of iron in excess. Additional IRE-containing mRNAs are controlled by IRPs. These proteins have crucial functions in iron homeostasis, such as the erythroid-specific enzyme of the heme biosynthetic pathway aminolevulinate synthase 2 and the iron transporters ferroportin 1 and divalent metal transporter 1 (DMT1; Hentze *et al.*, 2004; Pantopoulos, 2004). It has been demonstrated that iron and other molecules such as nitric oxide (NO) regulate IRE-binding activity in various cell types (Drapier *et al.*, 1993; Weiss *et al.*, 1993), and thus NO has a role in maintining iron homeostasis in mammal cells.

NO is a small, highly diffusible and ubiquitous bioactive molecule (Stamler *et al.*, 1992). It is recognized in animals as a biological mediator that plays important roles in key physiological processes such as maintaining blood pressure in the

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cardiovascular system, stimulating host defences in the immune system and regulating neural transmission in the brain (Schmidt and Walter, 1994).

In the last decade, considerable progress has been made in understanding the roles of NO in plants. NO functions as a ubiquitous signal involved in diverse physiological processes that include germination, root development, stomatal closing, induction of antioxidant enzymes, and adaptive responses to biotic and abiotic stresses (Lamattina *et al.*, 2003; Desikan *et al.*, 2004; Wendehenne *et al.*, 2004; Delledonne, 2005). In addition, recent data support a role for NO in plant iron homeostasis. Strong interactions between NO and iron, and the influence of NO on iron homeostasis in plant cells has been reported (Graziano and Lamattina, 2005). Various studies have established NO as a new player in plant iron metabolism (Murgia *et al.*, 2002; Graziano and Lamattina, 2005, 2007*a*; Arnaud *et al.*, 2006). New findings involving NO, the protein frataxin and iron metabolism in plant mitochondria (Martin *et al.*, 2009) are presented and discussed in the present review.

Overall, this review summarizes different aspects of iron homeostasis in plants and highlights possible directions of research, mainly those focused on a deep understanding of how NO biology influences iron metabolism in plants.

CHEMISTRY OF THE NO–IRON INTERACTION

NO presents different redox-related states, each of which is associated with specific reactions. These forms of NO include the uncharged nitric oxide ($NO^{\cdot}$), nitroxyl anion (NO^{-}) and the nitrosonium cation (NO^{+}) (Stamler *et al.*, 1992). $NO^{\cdot}$ reacts with metals to form NO^{+}, which then reacts with thiol groups to form *S*-nitrosothiols (e.g. *S*-nitrosocysteine, *S*-nitrosoglutathione and *S*-nitrosoalbumin) (Watts and Richardson, 2002). In biological systems, *S*-nitrosothiols have been proposed to prolong the half-life of NO, and to be the major storage and transport form of NO (Arnelle and Stamler, 1995; Lipton *et al.*, 2001; Pawloski *et al.*, 2001). The high affinity of NO for iron has been well characterized (Stamler *et al.*, 1992). Indeed, many of the biological effects of NO can be attributed to the fact that NO avidly binds iron within the active sites of numerous proteins. Some examples are the rate-limiting enzyme in DNA synthesis ribonucleotide reductase (Lepoivre *et al.*, 1991), ferritin (Lee *et al.*, 1994), haem-containing proteins (Ignarro, 1991; Khatsenko *et al.*, 1993; Griscavage *et al.*, 1994), proteins with [Fe–S] clusters involved in energy metabolism such as mitochondrial aconitase, and those that are part of complex I and II of the electron transport chain (Drapier and Hibbs, 1986, 1988). In terms of iron metabolism, one of the most important examples is the effect of NO on IRPs (see below). Furthermore, it has been shown that mononitrosyl–iron complexes (MNICs) or dinitrosyl–iron complexes (DNICs) form through the interaction between NO, iron and proteins or low-molecular-weight molecules (gluthatione or cysteine) containing thiols (Vanin, 1998). DNICs have the generic structure $[(RS^{-})_2\ Fe^{+}\ (NO^{+})_2]^{+}$, where the unpaired electrons of the nitrosyl ligands are transferred to the iron atom (Vanin and van Faassen, 2007*a*). Nitrite anions can induce DNIC formation in animal tissues and microorganisms. Initially, this was attributed to nitrite acidification and the subsequent release of NO from nitrous acid, but it is now known that some heme iron proteins and xanthine oxidase can also reduce nitrite to NO (Vanin and van Faassen, 2007*b*). However, DNICs may also be formed from NO radicals produced by the enzyme nitric oxide synthase (NOS). Formation of DNICs was not observed in animals treated with the NOS inhibitor *N*-nitro-L-arginine (Vanin and van Faassen, 2007*b*). Superoxide radicals (O_2^{-}) and peroxynitrite ($ONOO^{-}$) produced by the fast reaction between NO and O_2^{-} can destroy DNICs in tissues, keeping the basal concentration below the detection limit of EPR (electron paramagnetic resonance) (Vanin and van Faassen, 2007*b*). Experimental data have suggested that [Fe–S] clusters are an important source of DNIC formation following disruption of the clusters by NO. However, it has been demonstrated that the 'labile iron pool' or 'free iron' is the main source of DNIC formation in animal tissues and yeast (Vanin and van Faassen, 2007*b*).

NO AND CELLULAR IRON METABOLISM

NO is involved in the cellular iron-sensing mechanism in mammals

Under normal iron conditions, IRP1 displays aconitase activity that converts citrate to isocitrate in the tricarboxylic acid cycle by a catalytic [4Fe–4S] cluster present in the protein (Beinert and Kennedy, 1993). However, in iron-deficient cells aconitase activity decreases because its [Fe–S] cluster is disassembled and IRP1 then functions as an RNA-binding protein. This reversible switch allows aconitase/IRP1 constantly to sense iron levels and adapts the cell's response to iron requirements. IRP2 accumulates in iron-deprived cells but is quickly directed for proteasomal degradation under normal iron conditions (Iwai *et al.*, 1998).

It has been demonstrated that the regulatory activities of IRP1 and IRP2 differ in animal tissues compared with cell lines (Meyron-Holtz *et al.*, 2004). In primary cultures of mouse embryonic fibroblasts, IRP1 activity appears to be the most important IRE-binding protein when cells are iron-depleted (Meyron-Holtz *et al.*, 2004). However, in liver lysates from iron-deficient animals, the IRE-binding activity of IRP2 increased in response to iron deficiency, whereas the IRE-binding activity and aconitase activity of IRP1 remained constant (Meyron-Holtz *et al.*, 2004). One interesting point is that when cell lines are cultured in atmospheric oxygen (21 %), oxygen concentration in mammalian tissues is close to 3–6 %. It has also been shown that IRP2 is an effective iron sensor in macrophages and lymphocytes cultured at oxygen concentrations ranging from 3 to 21 %, whereas IRP1 is a poor sensor at low, physiological oxygen tensions.

IRPs are not only regulated by iron but also by other stimuli, including hypoxia (Hanson *et al.*, 1999), phosphorylation (Brown *et al.*, 1998; Fillebeen *et al.*, 2003), ROS (Hentze and Kühn, 1996; Pantopoulos and Hentze, 1998) and NO (Hentze and Kühn, 1996; Soum *et al.*, 2003). Two hypotheses have been put forward to explain NO-induced IRP1 activation. IRP1 can be rapidly activated by NO through direct interaction with its [4Fe–4S] cluster. Alternatively, NO, like the iron

chelator desferrioxamine, may have an indirect effect on IRP1 RNA-binding activity by depleting intracellular iron (Pantopoulos *et al.*, 1996; Wardrop *et al.*, 2000).

In contrast to IRP1, IRP2 does not contain the iron–sulfur cluster and it is degraded under iron-sufficient conditions. The role of NO in IRP2 regulation has been extensively studied and the diverse experimental systems have yielded conflicting results on whether NO activates or inhibits IRP2. In murine macrophages, it was demonstrated that IRP2 is *S*-nitrosylated by the NO donor sodium nitroprusside (SNP) and that this nitrosylation leads to its degradation via the ubiquitin-proteasome pathway (Kim *et al.*, 2004). By contrast, treatments of mouse B6 fibroblasts or human lung cancer cells with the NO-releasing drug *S*-nitroso-*N*-acetyl-penicillamine (SNAP) activate IRP2 expression (Wang *et al.*, 2005). These paradoxal results may be explained by the fact that NO possesses different biological effects depending on its redox state. NO in its reduced form, $NO^{\cdot}$, has high affinity for iron, whereas NO in its oxidized form, NO^{+}, causes *S*-nitrosylation of thiol groups of various proteins (Lipton *et al.*, 1993). Therefore, the roles of NO in mediating the regulation of iron metabolism can depend on the source of NO, the redox status of the cell and the amount of NO generated.

NO-mediated iron export from cells

Huge amounts of NO are produced by macrophages, which act as a cytotoxic effector that mediate iron release from tumour cells (Stuehr and Nathan, 1989; Stamler *et al.*, 1992; Richardson and Ponka, 1997). It has been shown that glucose metabolism and the subsequent generation of glutathione (GSH) enhanced the NO-mediated iron efflux from cells (Watts and Richardson, 2001, 2002, 2004). More recently, it was demonstrated that NO stimulates GSH and iron efflux from cells by active transport via a multi-drug resistance-associated protein 1 (MRP1), which belongs to the ABC family of transporters (Watts *et al.*, 2006). NO-mediated ^{59}Fe and GSH efflux is greater in cell types overexpressing MRP1. Treatments with buthionine sulphoximine (BSO), an inhibitor of GSH synthesis, and MRP1 inhibitors prevented NO-mediated ^{59}Fe and GSH efflux. Intracellular accumulation of DNICs was detected in treatments with MRP1 inhibitors. The tumour cells overexpressing MRP1 were found to be more sensitive to the anti-proliferative effects of NO than were wild-type cells. Thus, iron and GSH release from tumour cells could be critical for their proliferation. Accordingly, when activated macrophages are co-cultured with tumour cells, they inhibit target-cell DNA synthesis in tumour cells and induce the release of 64 % of cellular ^{59}Fe within 24 h (Hibbs *et al.*, 1984). The tumoricidal action of macrophage-derived NO may be due to the inhibition of iron-containing enzymes and involves repression of DNA synthesis, mitochondrial respiration and inactivation of the enzymes of the citric acid cycle in target cells (MacMicking *et al.*, 1997). Additionally, it is well known that cell iron mobilization by chelators results in anti-tumour activity (Richardson *et al.*, 1995). Therefore, the action of NO leading to iron and GSH release may be implicated in the cytotoxicity of macrophages against tumours (Watts *et al.*, 2006).

IRON ACQUISITION IN PLANTS

Iron nutrition is an important aspect of plant growth and development. Dynamic adjustments of the iron balance are required when iron levels fluctuate. Iron storage and buffering at sub-cellular level are critical mechanisms that allow plants to cope with iron insufficiency or toxicity due to iron overload. These processes involve a complex network of protein machineries (Briat *et al.*, 2007).

Iron is an abundant element. However, as the K_{sp} (solubility product constant) of $Fe(OH)_3$ is 10^{-38} M, the iron solubility in neutral or calcareous soils is very low, about 10^{-17} M at physiological pH. Iron concentrations required by plants are between 10^{-8} and 10^{-4} M, and therefore iron concentrations in alkaline soils are far below that those required for appropriate plant growth (Hell and Stephan, 2003). Oppositely, in acidic soils, excess ferric ion can be toxic to plants. Iron can operate as an oxidant factor because it catalyses generation of radical in the presence of reductants and peroxides. In particular, ferric ion is reduced by O_2^{-} to produce ferrous ion, which can react with H_2O_2 to form $OH^{\cdot}$ (Fenton reaction) (Ranieri *et al.*, 2001). Thus, iron uptake and transport have evolved in different ways in plants to be tightly regulated in order to provide adequate amounts for optimal growth, thus preventing excess accumulation (Kim and Guerinot, 2007).

Iron acquisition and transport within the plant

When dicotyledonous and monocotyledonous non-graminaceous plants are exposed to iron-deficient conditions they extrude protons into the rhizosphere through the activation of a specific plasma membrane H^{+}-ATPase of the root epidermal cells. This response induces rhizosphere acidification and, consequently, enhanced ferric iron solubilization. An H^{+}-ATPase gene, *CsHA1*, has been isolated from *Cucumis sativus* roots (Santi *et al.*, 2005), which seem to be specifically involved in iron-deficiency-induced acidification (Santi and Schmidt, 2008). CsHA1 localization was immuno-detected in epidermal, endodermal, cortical and root hair cells (Dell'Orto *et al.*, 2002). Interestingly, several H^{+}-ATPases that are members of the AHA (*Arabidopsis thaliana* H^{+}-ATPase) family are suggested to be involved in rhizosphere acidification. For instance, AHA7 is upregulated in response to iron deficiency (Colangelo and Guerinot, 2004). In *Solanum lycopersicum*, H^{+}-ATPase protein accumulation was shown in transfer cells formed in the rhizodermis of iron-deficient plants (Schmidt *et al.*, 2003). A specific root ferric chelate reductase (FRO) of the plasma membrane reduces ferric ion chelates to soluble ferrous ions before its transportation across the root cells (Robinson *et al.*, 1999). In *Arabidopsis*, the FRO family of metal reductases contains eight members (Wu *et al.*, 2005; Mukherjee *et al.*, 2006). *AtFRO2* expression is induced in epidermal cells of iron-deficient roots and is thought to be the main ferric chelate reductase in roots. The expression of *FRO* genes in various locations suggests different sets of FRO proteins involved in iron uptake in different plant tissues. In tomato, FRO1 mRNA is detected both in roots and in shoots and the protein is targeted to the plasma membrane. It is induced in response to iron deficiency in roots but not in leaves where

it is constitutively expressed (Eckhardt *et al.*, 2001). The ferrous ion generated is taken up by an iron-regulated transporter (IRT) belonging to the ZIP family. IRT1 appears to represent the major route for iron entering the cell, as indicated by the lethal chlorotic phenotype of *irt1*-knockout mutants (Henriques *et al.*, 2002; Varotto *et al.*, 2002; Vert *et al.*, 2002). Pulse labelling with ^{59}Fe through the root system shows that the *irt1* mutation reduces iron accumulation in the shoots. Short-term labelling with ^{65}Zn reveals a lower level of zinc accumulation, indicating that IRT1 can transport other divalent metals (Henriques *et al.*, 2002). The ZIP family of *Arabidopsis* contains 14 other members in addition to IRT1, with overall amino acid sequence similarities ranging between 38 and 85 %.

PLANT RESPONSES TO IRON DEFICIENCY

As stated above, roots of plants growing under iron-deficient conditions induce the expression of plasma membrane H^+-ATPase, ferric chelate reductase activities and ferrous ion transporters whereas these genes are repressed upon sufficient iron supply (Eide *et al.*, 1996; Robinson *et al.*, 1999; Vert *et al.*, 2002). The most important regulator in this process has been identified in tomato as the basic helix–loop–helix (bHLH) factor FER. T3238*fer*, an insertion mutant of *FER*, is unable to turn on the iron deficiency responses under iron-deficient stress. This mutant exhibits strong chlorosis and dies at early stage under normal culture conditions (Ling *et al.*, 2002). Using microarray analysis, a bHLH transcription factor FIT1 that regulates iron deficiency responses in *Arabidopsis* has been identified. FIT1 is required for proper regulation of ferric chelate reductase activity and iron transport into the plant root. FER and FIT1 share higher sequence similarity with each other than with any other known bHLH protein from the two species. *FIT1* can completely complement the malfunctions of the *fer* mutant when expressed in T3238*fer* (Yuan *et al.*, 2005). The *FIT1*-null mutants *fit1-1* and *fit1-2* (Colangelo and Guerinot, 2004) displayed similar iron deficiency symptoms as T3238*fer* and showed strong growth impairment. FIT1 regulates the ferric iron chelate reductase FRO2 at the level of mRNA accumulation and controls IRT1 protein accumulation. FIT1 can also control other genes implicated in iron homeostasis. In *35S:FIT1* plants, expression of target genes was not altered. This is probably because bHLH transcription factors have been shown to dimerize with other bHLHs and with members of other transcription factor families, such as the MYB family (Goff *et al.*, 1992; Abe *et al.*, 1997; Grotewold *et al.*, 2000), and both partners are probably required to affect transcription of target genes. It was recently demonstrated that FIT interacted with AtbHLH38 or AtbHLH39 to directly confer transcription activation of the iron uptake genes *FRO2* and *IRT1* (Yuan *et al.*, 2008). Apart from FIT (AtbHLH29), *AtbHLH38*, *AtbHLH39*, *AtbHLH100* and *AtbHLH101* also displayed upregulated transcription in roots and leaves under iron-deficient conditions (Wang *et al.*, 2007). Overexpression of *AtbHLH38* or *AtbHLH39* with *FIT* in *Arabidopsis* changed the expression pattern of *FRO2* and *IRT1* from induced to constitutive activation. More significantly, these transgenic plants were more tolerant to iron deficiency and accumulated more iron in their shoots (Yuan *et al.*, 2008).

More detailed and comprehensive updates regarding the adaptive responses of graminaceous plants to iron deficiency are presented in other contributions within this Highlight section.

A ROLE FOR NO IN THE REGULATION OF PLANT IRON METABOLISM

As in mammals, NO has been shown to be involved in iron signalling and metabolism in plants. Graziano *et al.* (2002) and Graziano and Lamattina (2007*a*) found strong evidence supporting a role for NO in recovering maize and tomato plants exposed to iron-deficient conditions. Figure 1 shows the effect of exogenous NO application on the recovery of maize and tomato seedlings growing under low iron concentration in nutrient solutions. Results obtained with iron-deficient maize plants suggest that the action of NO is related to improved iron availability inside the plant (Graziano *et al.*, 2002). Under iron-deficient growth conditions, NO treatment increased the chlorophyll content of leaves compared with in untreated plants, achieving similar chlorophyll levels to those found in maize plants growing in iron-sufficient conditions. NO-mediated chlorophyll increase was accompanied by the accumulation of transcripts encoding both the D1 protein of photosystem II and the Rubisco large subunit. Previous reports have shown that levels of these transcripts were

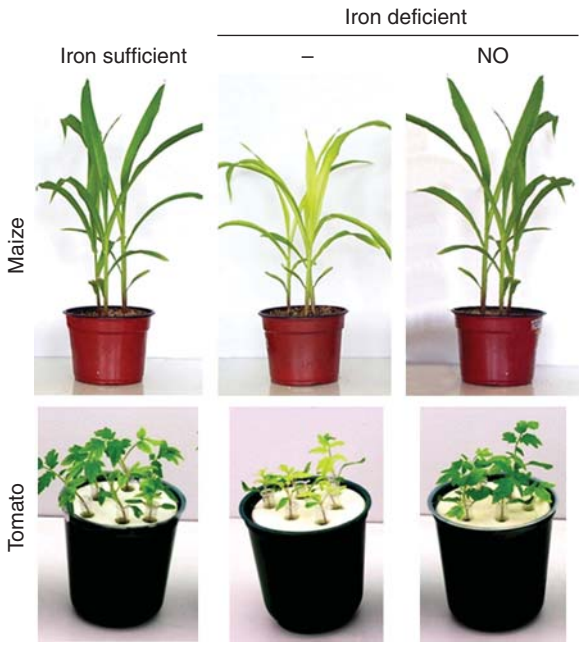

FIG. 1. Nitric oxide improves fitness of plants growing under low iron concentrations. Maize seedlings were grown in vermiculite watered with complete Hoagland's solution containing 200 μM Fe-EDTA (iron sufficient) or in Hoagland's solution containing 10 μM Fe-EDTA (iron deficient) with or without addition of 100 μM of the NO donor sodium nitroprusside (SNP) (NO^+). Tomato seedlings were grown in hydroponic conditions with complete Hoagland's solution containing 200 μM Fe-EDTA (iron sufficient) or in Hoagland's solution containing 10 μM Fe-EDTA (iron deficient) with or without addition of 100 μM of the NO donor nitrosoglutathione (GSNO) (NO^-).

reduced under iron deficiency and recovered after iron supply (Spiller *et al.*, 1987). However, iron concentrations in leaves, stems and roots were similar in both NO-treated and untreated maize plants growing under iron-deficient conditions (Graziano *et al.*, 2002). NO was also able to revert the phenotype of two iron-inefficient maize mutants, *ys1* and *ys3*, both impaired in iron uptake mechanisms. The *ys1* mutant holds the mutation on the protein YS1 involved in iron–phytosiderophore (PS) uptake (Curie *et al.*, 2001), whereas *ys3* is probably defective in PS secretion (Motta *et al.*, 2001). Sun *et al.* (2006) observed an increased content of thiobarbituric acid-reacting substances (TBARS) and decreased contents of protein-bound thiol (PT) and non-protein bound thiol (NPT) in maize growing in iron-deficiency conditions. These parameters are indicative of an oxidative damage on proteins and lipids. Interestingly, the NO donor SNP reduced the content of H_2O_2, O_2^- and TBARS, and increased PT and NPT levels, indicating that NO alleviates the iron deficiency-induced oxidative damage (Sun *et al.*, 2006). The activities of superoxide dismutase and glutathione reductase decreased sharply while the activities of catalase, peroxidase and ascorbate peroxidase increased after SNP treatment of iron-deficient maize plants. These data suggest that NO can protect maize plants from iron deficiency-induced oxidative stress by either reacting directly with ROS or by modulating activities of ROS-scavenging enzymes (Sun *et al.*, 2006). The role of NO in tomato plants growing under iron-deficient conditions has been also studied (Graziano and Lamattina, 2007*a*). The NO scavenger cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt) prevented the upregulation of *FER*, *FRO1* and *IRT1* genes in tomato plants growing under iron deprivation. Accordingly, exogenous application of the NO donor *S*-nitrosoglutathione (GSNO) enhanced the accumulation of *FER*, *FRO1* and *IRT1* mRNA in roots of iron-deficient tomato plants. NO was ineffective in inducing iron-deficiency responses in the tomato *fer* mutant, which indicates that the FER protein is necessary to mediate the action of NO. Furthermore, NO supplementation improved root hair development and plant growth under low iron supply, which suggests that NO is a key component of the regulatory mechanisms that control iron uptake and homeostasis in tomato. Moreover, exogenous application of NO can significantly improve the fitness of tomato plants growing under iron-deficient conditions (Graziano and Lamattina, 2007*a*). Taken together, the results indicate that NO contributes to the improvement of iron availability by (1) modulating the expression of iron uptake-related genes and (2) regulating the physiological and morphological adaptive responses of roots to iron-deficient conditions. These results are supported by the study of plant responses exposed to heavy metals. Cadmium (Cd^{2+}) is a non-essential metal displaying toxic effects in plants. It was shown that Cd^{2+} induces NO production in wheat (*Triticum aestivum*) roots (Groppa *et al.*, 2008) and in *Arabidopsis* leaves and roots (Besson-Bard *et al.*, 2009). It was shown that Cd^{2+}-mediated NO synthesis contributes to metal toxicity by inhibiting root growth. Besson-Bard *et al.* (2009) found that Cd^{2+}-induced NO production promotes IRT1 expression and Cd^{2+} overaccumulation in roots. They conclude that NO contributes to Cd^{2+} toxicity by favouring Cd^{2+} versus Ca^{2+} uptake and by activating a plant response resembling those occurring under iron deprivation.

As plants growing under iron deficiency do not show increased total iron content when they are supplemented with NO, it has been proposed that NO promotes iron re-mobilization in plants, as reported in animals cells (Vanin *et al.*, 2004; Graziano and Lamattina, 2005, 2007*b*). As the GSH level increases during iron deficiency (Zaharieva and Abadía, 2003) and NO accumulates under iron-deficient conditions (Graziano and Lamttina, 2007*a*), it is possible that NO could be mediating an increase in the available iron through DNIC formation. The DNIC-mediated increase of iron mobilization from roots to leaves improves basal metabolism as photosynthesis and normal growth conditions to be reached even at low iron supply (Graziano and Lamattina, 2007*b*). Furthermore, the formation of DNICs has been demonstrated in leaves from bean and China rose during treatments with gaseous NO (Vanin *et al.*, 2004). It has been observed that DNIC formation is induced in plants when endogenous NO production is stimulated or exogenously applied (Vanin *et al.*, 2004).

More recently, it has been postulated that phytohormone signals are probably the first step in transmiting the perception of iron deficiency. Thus, a hormone stimulus following the perception of low iron would induce cross-talk between NO, iron and GSH. The formation of DNICs could be operating to mobilize iron more efficiently and to establish a new balance in iron-deprived plants (Ramírez *et al.*, 2008). In summary, the data suggest DNICs are synthesized in plants and contribute not only to iron mobilization but also to deliver NO to differents plant organs.

Plant and animal ferritins have important similarities in their primary sequence as well as in their secondary and tertiary structures. Plant ferritins are found within plastids (Andrews *et al.*, 1992) and mitochondria (Zancani *et al.*, 2004, 2007). Whereas iron-induced ferritin synthesis is mainly controlled at the translational level in animals, transcriptional regulation of ferritin has been shown to be the main target of the iron overload response in plants (Klausner and Harford, 1989; Proudhon *et al.*, 1989; Theil, 1990; Lescure *et al.*, 1991). In maize, two independent pathways involved in the iron-mediated induction of ferritin synthesis have been demonstrated. One of these pathways involves the plant hormone abscisic acid (ABA) and the second pathway implicates reactive oxygen intermediates (Lobréaux *et al.*, 1993, 1995). Four ferritin genes have been identified in *Arabidopsis*, namely *AtFer1*, *AtFer2*, *AtFer3* and *AtFer4* (Gaymard *et al.*, 1996). In response to treatment with iron excess, an increase in the abundance of the *AtFer1* transcript was observed and, at later times, also of *AtFer3* and *AtFer4* transcripts (Petit *et al.*, 2001*a*). The *AtFer1* gene is positively regulated by H_2O_2 treatment, but it does not respond to treatment with exogenous ABA. *AtFer2* is the only *Arabidopsis* ferritin gene that is responsive to ABA treatment. It was suggested that AtFer4 could be a candidate for mitochondrial localization (Zancani *et al.*, 2004) although conclusive experimental evidence is still required. More recently, a dual targeting of Fer4 in plastids and mitochondria was also suggested (Martin *et al.*, 2009).

As already stated, animal cytosolic aconitase is known to regulate iron homeostasis through the IRE/IRP system. In

contrast, plant aconitases have not yet been shown to be functional IRPs. In *Arabidopsis* plants, three IRP1 homologues have been identified. The loss-of-function mutants in these genes showed a clear decrease in cytosolic aconitase activity. However, none of these mutants was affected in level of the ferritin transcript or protein accumulation in response to iron excess (Arnaud *et al.*, 2007). Also, recombinant tobacco and *Arabidopsis* aconitases failed to bind to IRE from the human ferritin transcript (Moeder *et al.*, 2007). The evidence suggests that, in plants, aconitases are not converted into a functional IRP. Navarre *et al.* (2000) found that tobacco aconitase is inactivated by NO, which may alter the iron balance by increasing free iron levels due to the NO-mediated destruction of the aconitase iron–sulfur cluster. Despite the fact that IRE-like sequences have not been detected in plants (Pesole *et al.*, 2000), a *cis*-acting element necessary for the iron-dependent regulation of the transcription of maize *ZmFer1* and *Arabidopsis AtFer1* ferritin has been found in the promoter sequences through experiments based on serial deletions and site-directed mutagenesis (Petit *et al.*, 2001*b*). This sequence, named IDRS, for iron-dependent regulatory sequence, has been shown to be involved in the repression of *ZmFer1* and *AtFer1* gene expression under iron-deficient conditions (Petit *et al.*, 2001*b*; Tarantino *et al.*, 2003). Sequences similar to the *Arabidopsis Fer1* IDRS element have been identified in the other three *Arabidopsis* ferritin genes (Petit *et al.*, 2001*b*). However, it remains to be demonstrated whether the IDRS-like sequences observed in the *AtFer2*, *AtFer3* and *AtFer4* promoter sequences are functional.

The NO donor SNP induces the accumulation of ferritin both at mRNA and protein level. The NO scavenger cPTIO prevents ferritin transcript accumulation in *Arabidopsis* cell suspension cultures treated with iron excess. As mentioned above, plant ferritin mRNA lacks the IRE sequence and thereby NO regulation of ferritin expression occurs in a different way to that described in animal systems. It was demonstrated that NO mediates plant ferritin regulation through the IDRS sequence of the *AtFer1* promoter, which is also an element responsible for transcriptional repression under low iron supply (Murgia *et al.*, 2002). Furthermore, iron overload leads to a rapid NO burst in cell plastids (Arnaud *et al.*, 2006). By contrast, experiments were performed to assay the effect of exogenous addition of NO in plant cell culture. The effects of different NO donors releasing different NO forms, NO^+ (SNP) or $NO^{\cdot}$ (SNAP, GSNO, NOC-18), have shown that SNP behaves differently from the other NO donors tested. It was found that SNP induces accumulation of ferritin transcripts in *Arabidopsis*, whereas SNAP inhibits its accumulation (Murgia *et al.*, 2004). These results suggest that the induction of ferritin is very sensitive to the influence of the redox status of the cell, which may determine the NO form. Figure 2 shows a scheme that represents the involvement of NO in plant responses to iron deficiency or to iron overload. As stated, NO is induced in both situations and triggers downstream signals. In iron-deficient conditions, NO seems to be a key molecule in the regulation of the well-characterized strategy I responses, including induction of the *FER* transcription factor, *FRO* and *IRT* expression in tomato. Under iron overload, NO generation is critical for the activation of ferritin expression. One interesting question is which are the molecular mechanisms triggering NO production upon changes in intracellular iron status?

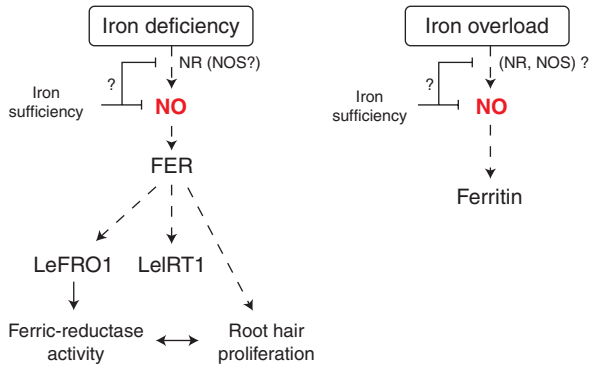

FIG. 2. Overview of nitric oxide-regulated genes involved in plant responses to alterations in iron nutrition. The diagram shows a very simple scheme of the known NO-promoted responses of strategy I plants growing in either iron deficiency or iron overload. NR, nitrate reductase; NOS, nitric oxide synthase; NO, nitric oxide; FER, bHLH transcription factor; FRO, Fe-chelate reductase; IRT, iron transporter.

FRATAXIN–NO INTERACTION CONTRIBUTES TO CONTROL CELLULAR IRON HOMEOSTASIS

Mammal and plant mitochondria are an essential and dynamic component of cellular biochemistry (Noctor *et al.*, 2007; Mackenzie *et al.*, 2008). It is known that iron plays a crucial role in mitochondrial metabolism and that the disruption of iron homeostasis can be catastrophic. However, our knowledge on trafficking, storage and metabolism of iron in mitochondria is still scarce.

Frataxin is a mitochondrial protein that is highly conserved in mammals, yeast, bacteria and plants. This strongly suggests that frataxin is an essential protein that could play similar roles in many unrelated organisms. In humans, mutations in the frataxin gene are responsible for the cardio-neuro degenerative disease Friedreich's ataxia (Napier *et al.*, 2005). Several functions have been proposed for frataxin including iron homeostasis (Foury and Talibi, 2001; Napier *et al.*, 2005), Fe–S cluster assembly (Chen *et al.*, 2002; Duby *et al.*, 2002; Lill and Muhlenhoff, 2005), regulation of respiration and oxidative phosphorylation (Ristow *et al.*, 2000; Santos *et al.*, 2004), control of antioxidant defences (Gakh *et al.*, 2006) and iron chaperone modulating mitochondrial aconitase activity (Bulteau *et al.*, 2004). In many organisms, frataxin deficiency is associated with iron accumulation in mitochondria and oxidative stress (Babcock *et al.*, 1997). Iron overload triggers oxidative stress and iron detoxification seems to be a relevant frataxin function in antioxidant defence. In that sense, frataxin deficiency has been shown to increase the sensitivity of yeast cells to oxidative stress (Babcock *et al.*, 1997; Foury and Cazzalini, 1997).

In plants, one *Arabidopsis* homologous gene (*AtFH*) has been identified (Busi *et al.*, 2004). This gene is able to complement null mutant frataxin yeast (Δ*yfh*), strongly indicating functional similarities between plant and yeast frataxin. Two knock-outs mutants and one knock-down T-DNA insertional mutant in *Arabidopsis* frataxin have been recently analysed.

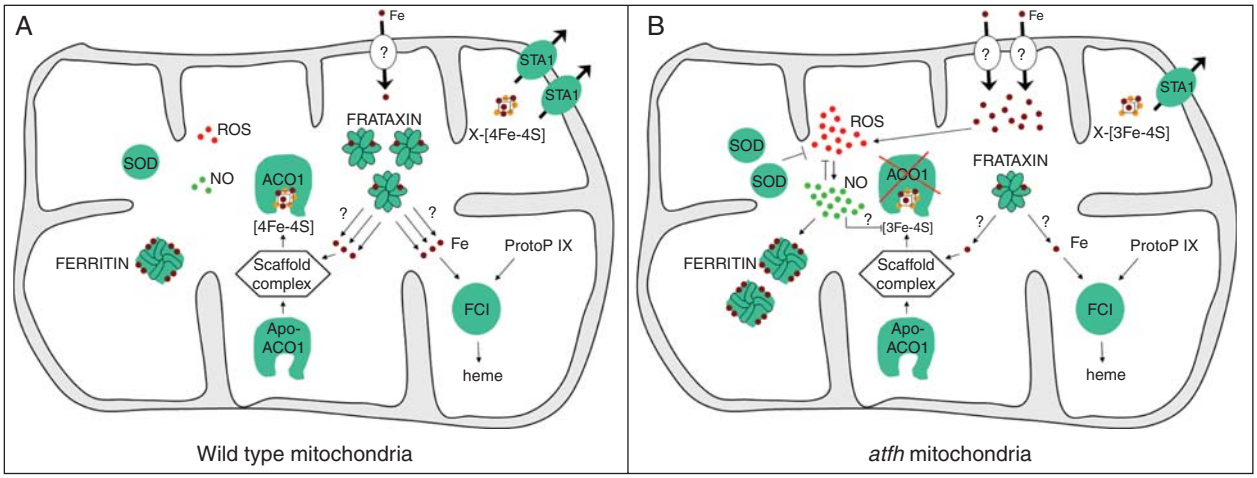

FIG. 3. Frataxin deficiency leads to increasing iron, ferritin, ROS and NO in plant mitochondria. Frataxin deficiency leads to iron (Fe, red–brown circles) accumulation. Free iron induces formation of reactive oxygen species (ROS, red circles) via the Fenton reaction and nitric oxide accumulation (NO, green circles), which may function as an ROS scavenger and triggers the induction of ferritin to diminish free iron. Abnormally high ROS contents induce also antioxidant enzymes such as superoxide dismutase (SOD). Reduction of frataxin also disrupts mitochondrial aconitase (ACO1) activity possibly through NO accumulation and/or defects in assembly of iron–sulfur clusters [Fe–S]. Clusters [4Fe–4S] are represented by an empty cube with red–brown circles (Fe) and orange circles (S, sulfur) in their vertices. Frataxin deficiency causes a decreased STA1 steady-state level, which might contribute to iron accumulation (M. Martin *et al.*, UNMdP, Argentina, unpubl. res.). Frataxin has also been implicated in heme biosynthesis interacting with ferrochelatase1 (FC1) activity (Yoon and Cowan, 2004). Protein symbols have been extracted from Briat *et al.* (2007).

The knock-out mutants (*atfh-2* and *atfh-3*) present an embryo lethal phenotype, indicating an essential role of frataxin at this developmental stage of seed formation (Busi *et al.*, 2006; Vazzola *et al.*, 2007). The knock-down mutant (*atfh-1*) plant has reduced frataxin mRNA and protein levels. In this mutant, the activity of two Fe–S-containing enzymes, mitochondrial aconitase and succinate dehydrogenase, is reduced whereas malate dehydrogenase, which does not contain the Fe–S moiety, remains almost unaltered, indicating a role of frataxin in Fe–S cluster assembly and/or insertion (Busi *et al.*, 2006). These results indicate that frataxin is an essential protein in plants, required for full activity of mitochondrial Fe–S proteins. The *atfh-1* mutant shows iron accumulation in mitochondria as well as in plastids (Martin *et al.*, 2009). Part of the iron accumulation observed in mitochondria could be due to a decrease in the steady-state level of STA1 transcript, a gene coding for an ABC transporter implicated in Fe–S cluster exportation from mitochondria (Kushnir *et al.*, 2001; M. Martin *et al.*, UNMdP, Argentina, unpubl. res.). These results suggest that decreased frataxin levels cause iron accumulation in plants, as was shown in other organisms (Babcock *et al.*, 1997; Napier *et al.*, 2005). It has been demonstrated that excess of free iron produces hydroxyl radicals ($OH^{\cdot}$) via the Fenton reaction (Halliwell and Gutteridge, 1992). Indeed, it was reported that frataxin-deficient plants exhibit increased ROS formation and induction of detoxifying enzymes involved in the oxidative stress response, indicating that *atfh-1* plants are under oxidative stress (Busi *et al.*, 2006). The abnormally high iron and ROS contents parallel an increase of NO production in both plants and yeast (Martin *et al.*, 2009). This increased NO production has been shown to be essential to maintain low levels of oxidative damage in root cells (Martin *et al.*, 2009). It has also been shown that accumulation of NO may provide protection from oxidative stress in two ways: (1) directly by scavenging peroxide (Beligni and Lamattina, 1999) and (2) indirectly via NO-mediated induction of ferritin genes (*AtFer1* and *AtFer4*) (Murgia *et al.*, 2002; Martin *et al.*, 2009). Thus, NO can positively contribute to diminish free iron levels within the organelles and it can control ROS formation with consequent protection from oxidative stress and cell death. It is then suggested that high NO production in *atfh-1* plants might be part of the response to iron-mediated oxidative stress caused by deficiency of frataxin. Figure 3 shows a model to explain changes in mitochondria of *atfh-1* plants. While mitochondrial homeostasis is maintained through a coordinated regulation of their components in wild-type *Arabidopsis* plants (Fig. 3A), frataxin deficiency leads to iron accumulation and to increased ROS and NO production. As a consequence, it is proposed that in *atfh-1* plants, ferritin expression is induced by NO to sequester the excess free iron (Fig. 3B). The formation of $ONOO^{-}$ after the rapid reaction between O_{2-} and NO could be responsible for inhibiting aconitase by disrupting its Fe–S cluster. In a direct way, frataxin deficiency also contributes to a disminished ability to function as an iron chaperone protein cluster, resulting in aconitase inactivation. Thus, the evidence indicates that ferritins, frataxin and NO appear to be essential components to maintain cellular iron homeostasis in plants through the control of mitochondrial iron homeostasis.

CONCLUDING REMARKS AND PERSPECTIVES

Several lines of evidence from chemical and biological approaches strongly support that NO is an active component of the physiological adaptive responses involved in maintaining iron homeostasis in plants and animals. The redox chemistry of iron and NO is tightly linked through the affinity of the free radical for the metal and both are regulators of the cell redox status. NO probably operates in a coordinated manner with the

ascorbate–glutathione cycle to control the antioxidant defence system pathways. Frataxin is a recently studied protein in plants which seems to be involved in iron metabolism through its activity as an iron chaperone protein on the Fe–S cluster assembly in mitochondrial proteins. However, its actual function remains poorly understood. Interestingly, NO production is promoted when frataxin is deficient. This frataxin deficiency-dependent NO production has been shown to be involved in: (1) inducing ferritin expression possibly to keep low levels of free iron in mitochondria, and (2) protecting mitochondrial functions and cell viability from damage produced by a high free iron-dependent increased oxidative stress.

Iron metabolism and free radical generation (NO, H_2O_2, $O_2^{\cdot-}$) are related to several human diseases such as Friedreich's ataxia, Huntington's, Parkinson's and Alzheimer's diseases, motor neuron disease (amyotrophic lateral sclerosis) and Wilson disease. Plant and animal ferritins and frataxin seem to share similar features in iron homeostasis and both are linked to NO generation and control of cellular redox state. *Arabidopsis* plants thus emerge as a serious and useful model to study treatments for these diseases and to provide an understanding of the molecular basis of the cross-talk between ferritin, frataxin and NO.

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