Journal of Experimental Botany, Vol. 72, No. 4 pp. 990–1006, 2021
doi:10.1093/jxb/eraa484 Advance Access Publication 16 November 2020

REVIEW PAPER

Evidence for a role of nitric oxide in iron homeostasis in plants

Rajesh Kumar Tewari[1], Nele Horemans[2,3], and Masami Watanabe[4]
[1] Department of Botany, University of Lucknow, Lucknow, Uttar Pradesh 226007, India
[2] Biosphere Impact Studies, Belgian Nuclear Research Center (SCK•CEN), Boeretang 200, 2400 Mol, Belgium
[3] Centre for Environmental Sciences, Hasselt University, Agoralaan gebouw D, 3590 Diepenbeek, Belgium
[4] Laboratory of Plant Biochemistry, Chiba University, Inage-ward, Yayoicho 1–33, Chiba, 263-8522, Japan

* Correspondence: rktewari_bot@yahoo.com

Received 26 August 2020; Editorial decision 7 October 2020; Accepted 13 October 2020

Editor: Karl-Josef Dietz, Bielefeld University, Germany

Abstract

Nitric oxide (NO), once regarded as a poisonous air pollutant, is now understood as a regulatory molecule essential for several biological functions in plants. In this review, we summarize NO generation in different plant organs and cellular compartments, and also discuss the role of NO in iron (Fe) homeostasis, particularly in Fe-deficient plants. Fe is one of the most limiting essential nutrient elements for plants. Plants often exhibit Fe deficiency symptoms despite sufficient tissue Fe concentrations. NO appears to not only up-regulate Fe uptake mechanisms but also makes Fe more bioavailable for metabolic functions. NO forms complexes with Fe, which can then be delivered into target cells/tissues. NO generated in plants can alleviate oxidative stress by regulating antioxidant defense processes, probably by improving functional Fe status and by inducing post-translational modifications in the enzymes/proteins involved in antioxidant defense responses. It is hypothesized that NO acts in cooperation with transcription factors such as bHLHs, FIT, and IRO to regulate the expression of enzymes and proteins essential for Fe homeostasis. However, further investigations are needed to disentangle the interaction of NO with intracellular target molecules that leads to enhanced internal Fe availability in plants.

Keywords: Antioxidant defense, functional iron, iron availability, iron-related enzymes, nitric oxide, reactive oxygen species.

Introduction

Iron (Fe) is an essential micronutrient for all living organisms, including plants. It is an integral component of many proteins required for crucial cellular processes and is involved in various vital functions, including photosynthesis, respiration, and cell division (Broadley *et al.*, 2012). Fe is abundant in the Earth's crust, constituting 5.1% by mass (Murad and Fischer, 1988; Guerinot, 2001), but is mostly present in insoluble forms and thus unavailable for uptake by plants. For example, in aerobic soils or at higher pH levels Fe is readily oxidized and will precipitate into insoluble Fe(III)-oxyhydroxide complexes. The molecular mechanisms that facilitate Fe acquisition in plants include solubilization of Fe^{3+}, complexation, and subsequent uptake from the soil and into root cells (Robinson *et al.*, 1999). Plants

have been shown to follow one of two alternative strategies for Fe uptake. In strategy I plant species, which include most dicot plants, the uptake of Fe is supported by acidification of the rhizosphere and reduction of external Fe(III) to Fe(II) (Guerinot and Yi, 1994; Broadley *et al.*, 2012). Monocot grasses (strategy II plants), on the other hand, chelate Fe^{3+} by releasing phytosiderophores, which are low-molecular-weight non-proteinogenic amino acids that function as Fe^{3+}-specific ligands (Guerinot and Yi, 1994; Guerinot, 2007). In some plant species, such as rice, both of these strategies coexist (Guerinot, 2007; Ishimaru *et al.*, 2007). Despite the existence of these Fe-uptake mechanisms, plants often exhibit typical interveinal chlorosis in young and emerging leaves, presumably when the physiologically active fraction of tissue Fe is less than the threshold Fe requirement for plants. In addition, the total Fe content of plants may often not be an index of functional Fe in the tissues (Kumar *et al.*, 2010; Tewari, 2019).

Nitric oxide (NO) is a biologically active, diatomic, diffusive, water- and lipid-soluble, gaseous free radical or molecule (Tewari *et al.*, 2019). NO is involved in cellular signaling processes at nanomolar concentrations (1.0 nmol l^{-1}). It has been demonstrated to be a ubiquitous signaling molecule in animals, plants, and microbial systems (Crane, 2008; Crane *et al.*, 2010). Initially, NO was considered to be an environmental pollutant (Delledonne, 2005). The discovery that NO acts as an essential regulator in biological systems has originated from three fundamental findings: (i) the identification of NO as a endothelial relaxing factor in the vascular system, (ii) the identification of NO as a key cytotoxic agent of the immune system, and (iii) the discovery of the function of NO as a signaling molecule in the nervous system (Crane, 2008; Crane *et al.*, 2010). This review focuses on plant NO-generating mechanisms and the potential role of NO in Fe homeostasis, particularly in Fe-deficient plants.

NO-generating mechanisms in plants

In mammals, three isozymes responsible for regulating active NO synthesis, namely endothelial nitric oxide synthase (eNOS or NOS-III), inducible NOS (iNOS or NOS-II), and neuronal NOS (nNOS or NOS-I), have been discovered (Crane *et al.*, 2010). These NOSs catalyze the conversion of L-arginine to L-citrulline and NO in the presence of O_2.

$$\text{L} - \text{Arginine} \xrightarrow[1.0\ \text{NADPH}+\text{O}_2\quad \text{H}_2\text{O}]{\text{NOS}} \text{N}^{\text{G}} - \text{hydroxy}$$

$$\text{L} - \text{Arginine} \xrightarrow[0.5\ \text{NADPH}+\text{O}_2\quad \text{H}_2\text{O}]{\text{NOS}} \text{L} - \text{Citrulline} + \text{NO}$$

Although there are several reports on NOS-like activity in plants (Neill *et al.*, 2003; Corpas et al., 2009, 2011); no functional NOS-like protein has yet been isolated and characterized from a higher plant system (Barroso *et al.*, 1999; Corpas *et al.*, 2001). However, molecular and biochemical studies have revealed the existence of a NOS-like sequence in the genome of two green algae, *Ostreococcus tauri* and *Ostreococcus lucimarinus* (Foresi *et al.*, 2010). In this respect, it was shown that *Escherichia coli* expressing recombinant *O. tauri* NOS had increased NO levels and cell viability (Foresi *et al.*, 2010). Further studies with *Arabidopsis thaliana* indicated the existence of a NOS-like protein in the peroxisomes of root tips (Corpas and Barroso, 2014a, b). Peroxisomal NOS is synthesized in the cytosol and transported to peroxisomes due to the presence of an N-terminal signal peptide, peroxisomal targeting signal type 2 (PTS2) (Corpas and Barroso, 2014b). Apart from NOS-catalyzed NO production, two other molecules, namely polyamines (Tun *et al.*, 2006) and hydroxylamine (Rumer *et al.*, 2009), also produce NO in plants. These reactions occur under normoxic conditions and are activated by stresses and reactive oxygen species (ROS) (Yamasaki and Cohen, 2006; Gupta *et al.*, 2011a).

Other potential enzymatic sources of NO in plants are nitrate reductase (NR) (Neill et al., 2003, 2008b) and xanthine oxidase-like (XOR-like) activity (Tewari *et al.*, 2009; Bender and Schwarz, 2018). NO is produced by the reduction of nitrite by heme- or Cu-containing nitrite reductases (NiRs) and is then further reduced to nitrous oxide (N_2O) by the NO reductases (NORs) (Crane *et al.*, 2010). In addition, a root-specific plasma membrane nitrite-NO reductase (Ni-NOR) is involved in NO formation from nitrite exclusively in roots. Ni-NOR activity is coordinated with a plasma membrane-bound NR that reduces nitrate to nitrite (Besson-Bard *et al.*, 2008; Astier *et al.*, 2017). In the unicellular alga *Chlamydomonas reinhardtii*, it has been shown that NR can interact with a new partner protein, NO-forming NiR (NOFNiR), to produce NO from nitrite (Chamizo-Ampudia *et al.*, 2017). XOR is capable of reducing organic nitrate as well as inorganic nitrate and nitrite. XOR releases NO using either NADH or xanthine as a reducing agent, particularly under anaerobic conditions (Godber *et al.*, 2000; Bender and Schwarz, 2018). Under aerobic conditions, XOR-catalyzed generation of NO is rather slow but still present (Godber *et al.*, 2000; Bender and Schwarz, 2018). Moreover, nitrite reacts with deoxyhemoglobin and produces NO. In this reaction, deoxyhemoglobin is oxidized to methemoglobin (Grubina *et al.*, 2007). Non-enzymatic production of NO from nitrite involving plastid pigments such as carotenoids (Cooney *et al.*, 1994), a reductant such as ascorbic acid, and acidic pH (Tewari *et al.*, 2009) have also been reported. Moreover, certain phenols (quercetin, epicatechin gallate, and epigallocatechin gallate) also produce NO, as shown in Fig. 1 (Appeldoorn *et al.*, 2009; Takahama and Hirota, 2017). In conclusion, probably two main pathways—the nitrite and the L-arginine pathway—are involved in NO generation in plants (Fig.1).

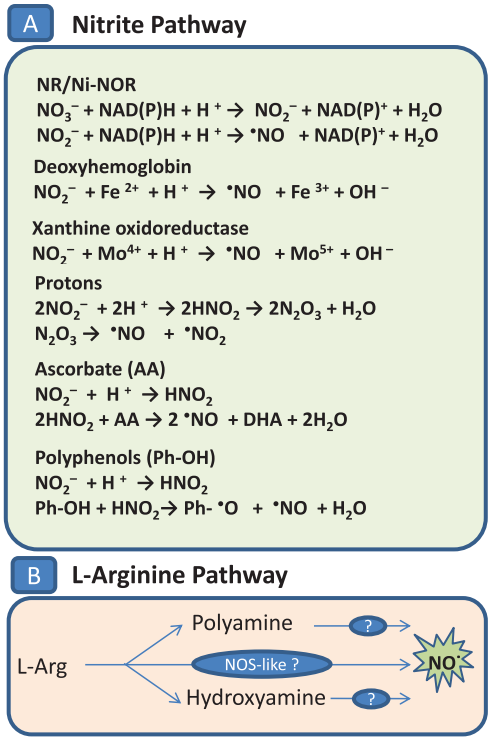

Fig. 1. NO generation pathways in plants. (A) Nitrite pathways involve various enzymes/proteins, such as NR/Ni-NOR (which respectively catalyze nitrate to nitrite and nitrite to NO), deoxyhemoglobin, and xanthine oxidoreductase, as well as protons, ascorbic acid, and polyphenols; these pathways utilize nitrite to produce NO in the plant cell. (B) The L-arginine pathway involves mostly NOS-like enzyme, polyamines, or hydroxylamine for NO synthesis. However, further studies are needed to decipher the biochemical nature of the NOS-like factor involved in these reactions.

Spatial sites of NO generation in plant tissue and cells

NO generation has been reported in various plant species and their organs (Fig. 2), for example, in the root, stem, and leaf of pea plants (Corpas et al., 2004, 2009), in Arabidopsis leaves and roots (Zhao *et al.*, 2007b; Ribeiro *et al.*, 2009), in maize roots and leaves (Zhang *et al.*, 2006b; Zhao *et al.*, 2007a; Sang *et al.*, 2008), in *Panax ginseng* adventitious roots under Cu excess (Tewari *et al.*, 2008b), and in senescing *Phalaenopsis* flower tepals (Tewari *et al.*, 2009). Additionally, NO generation has been observed in pea leaf vascular tissues, xylem, and phloem (Corpas *et al.*, 2004), and in differentiating xylem of *Zinnia elegans* (Gabaldón *et al.*, 2004; Novo-Uzal *et al.*, 2013).

At a subcellular level, NO generation occurs both in the apoplast (the space outside the plasma membrane, including the cell wall) (Bethke *et al.*, 2004) and in the symplast (the space inside the plasma membrane, including the cellular organelles) (Tewari *et al.*, 2013b; Feigl *et al.*, 2014). NO generation has been reported in various subcellular compartments (Fig. 3), such as rapeseed leaf cytoplasm (Yasuda *et al.*, 2007), pea leaf peroxisomes (Barroso *et al.*, 1999; Corpas *et al.*, 2004; Feigl *et al.*, 2014), pollen tube peroxisomes of lily (Prado *et al.*, 2004), barley root mitochondria in the presence of nitrite and NADH (Gupta and Kaiser, 2010; Gupta et al., 2011b, 2020b), soybean leaf chloroplasts in the presence of arginine, $NaNO_2$, and *S*-nitrosoglutathione (GSNO) (Jasid *et al.*, 2006), rapeseed leaf mesophyll chloroplasts (Tewari *et al.*, 2013b), and soybean cotyledon chloroplasts (Galatro *et al.*, 2013) in the absence of NO-producing chemicals.

NO regulates various physiological and developmental processes in plants

In animal systems, NO has been shown to be a biological mediator that plays central roles in key physiological processes such as neurotransmission, immunological and inflammatory responses, and the relaxation of vascular smooth muscle tissue (Besson-Bard *et al.*, 2008). In plants, NO has already been demonstrated to be involved in a variety of physiological processes, including seed germination (Kopyra and Gwóźdź, 2003; Sarath *et al.*, 2005; Piterkova *et al.*, 2012) and the induction of lateral roots (Creus *et al.*, 2005; Tewari *et al.*, 2008a). NO delays senescence (Neill *et al.*, 2003), regulates fruit ripening (Corpas *et al.*, 2018; González-Gordo *et al.*, 2019) and stomatal movement (Neill *et al.*, 2008a), and directs targeted (oriented) growth of pollen tubes towards ovules in *Lilium logiflorum* (Prado *et al.*, 2004; Shah *et al.*, 2019) and Arabidopsis (Prado *et al.*, 2008). It has been observed that differentiating xylem cells generally exhibit relatively higher NO production compared with fully differentiated xylem cells. This observation suggests that NO participates in xylem differentiation (Gabaldón *et al.*, 2004; Novo-Uzal *et al.*, 2013). NO also participates in cell wall lignification by modulating the activities of basic peroxidases (Novo-Uzal *et al.*, 2013). Moreover, NO application has been reported to be involved in the biosynthesis of secondary metabolites, as it stimulates phenylalanine ammonia lyase and the accumulation of phenols (Kovacik *et al.*, 2009), and appears to be involved in low-energy ultrasound- and methyl jasmonate-induced taxol biosynthesis in *Taxus* cell suspension culture (Wang and Wu, 2005; Wang *et al.*, 2006) and salicylic-acid induced synthesis of ginsenosides in the adventitious roots of *P. ginseng* (Tewari and Paek, 2011).

NO improves the functional Fe status of plants and prevents Fe chlorosis under Fe-deficient conditions

NO supply using NO-producing molecules such as sodium nitroprusside (SNP), *S*-nitroso-*N*-acetylpenicillamine (SNAP), and GSNO has been reported to prevent interveinal chlorosis (a typical symptom of Fe deficiency) even in plants supplied with very low (10 μM) Fe (Graziano *et al.*, 2002; Graziano and Lamattina, 2005, 2007; Sun *et al.*, 2007; Kumar

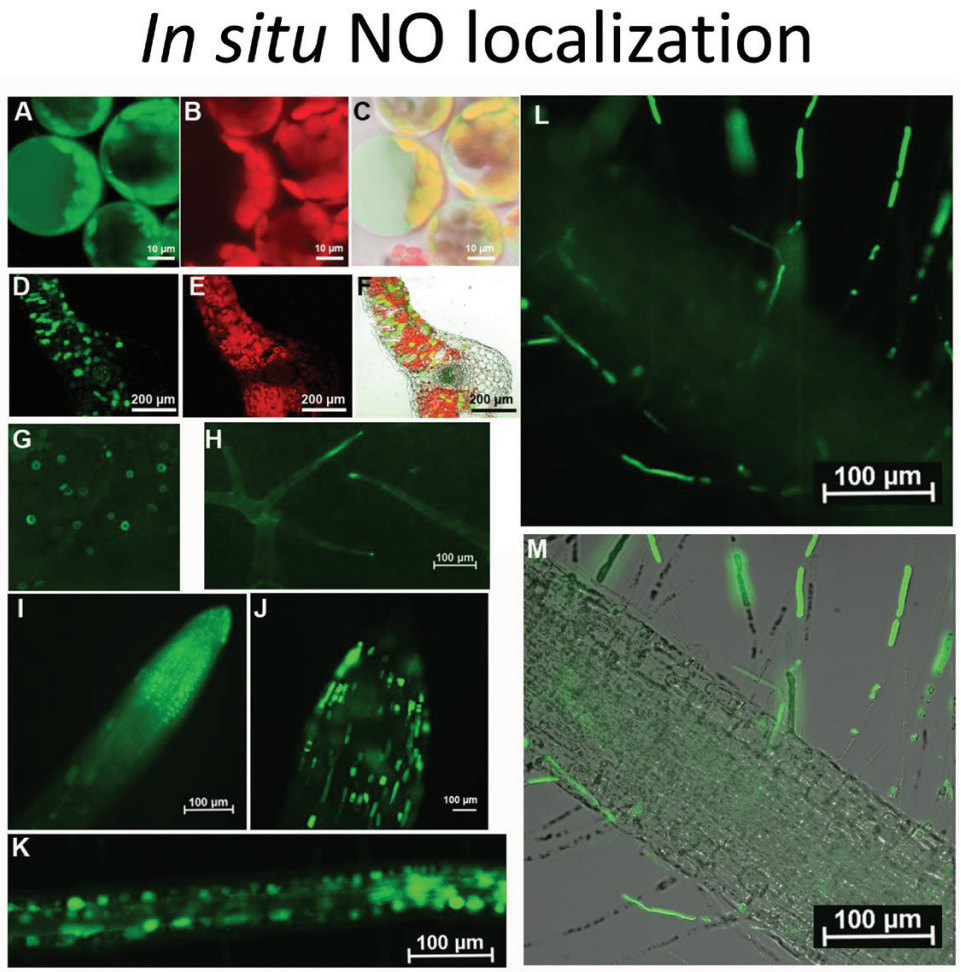

Fig. 2. NO localization using diaminofluorescein-2 diacetate (DAF-2DA) in various cells and tissues. (A) 4,5-Diaminofluorescein triazole (DAF-2T) is the reaction product of DAF-2DA with NO, represented by green fluorescence (an indicator of NO production) in protoplast chloroplasts. (B) Chlorophyll fluorescence (red) in the same protoplasts. (C) Merged image of (A) and (B), showing green fluorescence in the chloroplasts. (D) DAF-2T fluorescence in mesophyll cells. (E) Chlorophyll fluorescence in the same mesophyll cells. (F) Merged image of (D) and (E), showing green fluorescence in the mesophyll cells. (G–L) DAF-2T fluorescence in (G) guard cells, (H) trichomes, (I) root apex, (J) epidermal cells of the root apex, (K) root, and (L) root hairs. (M) Merged green fluorescence and visible light image showing DAF-2T fluorescence in root hairs.

et al., 2010). Exposure to gaseous NO also produced a similar effect in Fe-deficient maize plants (Graziano *et al.*, 2002). Exposure to the NO producers SNP and DETA NONOate is claimed to increase the labile Fe pool in sorghum plants (Jasid *et al.*, 2008; Robello *et al.*, 2019). Fe-deficient plants treated with SNP along with NO scavengers [(2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) or methylene blue] did not exhibit recovery from the effects of Fe deficiency. Moreover, an equimolar concentration of sodium ferrocyanide, the molecular structure of which contains an atom of Fe, failed to improve the chlorophyll status of the leaves of Fe-deficient plants. This observation suggests that the observed recovery in Fe deficiency was caused by NO supply. Graziano *et al.* (2002) found that the effect of SNP-mediated NO supply on the recovery of plants from Fe deficiency was comparable to that of NO supplied as GSNO or directly as gaseous NO. Greening of foliage of the NO-treated Fe-deficient maize plants was accompanied with increases in the chlorophyll concentration (Graziano *et al.*, 2002; Kumar *et al.*, 2010). However, the application of SNP as a NO producer did not appear to be very realistic in order to study the role of NO in Fe nutrition, as this molecule itself contains an Fe atom. In conclusion, the findings reported in these studies together suggest that NO either enhanced the amounts of functional Fe under Fe-deficient conditions or diminished the threshold level of Fe required by plants to grow normally. However, since the total Fe concentration of the leaf showed no direct correlation with the concentration of chlorophyll or carotenoids, NO induced regreening of chlorotic leaves by increasing the functionally available Fe levels (Kumar *et al.*, 2010). In contrast, Song *et al.* (2016) observed an increased concentration of total Fe in Fe-deficient peanut plants supplied with 250 µM SNP. It may be concluded that NO mediated improvement in the functional Fe status in Fe-deficient plants by enhancing the

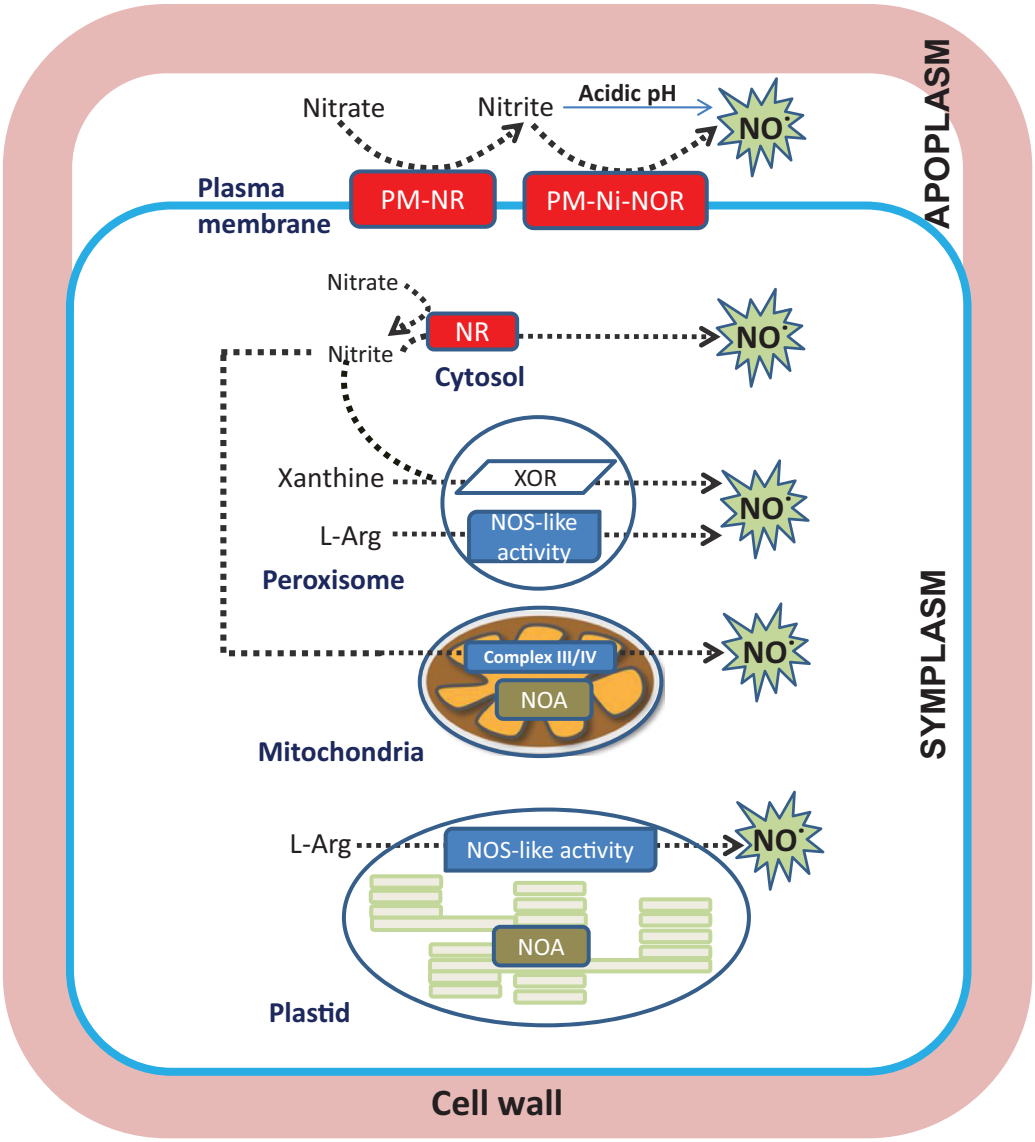

Fig. 3. Schematic representation of an extracellular compartment (apoplastic location) and the subcellular compartments (symplastic locations) in which NO generation through the nitrite or L-arginine pathways has been reported. NOA, Nitric oxide associated 1. Redrawn after Gupta *et al.* (2011a). On the origins of nitric oxide. Trends in Plant Science 16, 160–168. Copyright (2011), with permission from Elsevier.

reduction of apoplastic Fe(III) to Fe(II) (Graziano *et al.*, 2002; Graziano and Lamattina, 2005).

NO supply has been shown to increase leaf chlorophyll content by de-etiolation (Beligni and Lamattina, 2001; Graziano and Lamattina, 2007), and NO appears to be crucially involved in the light-mediated greening of seedlings (Zhang *et al.*, 2006a). NO-mediated greening of etiolated seedlings appears to be related to an internal increase in functional Fe status. NO-mediated improvement of the functional Fe status of plants growing under Fe-deficient conditions represents a useful tool to cope with the low Fe availability of calcareous soils (Lamattina *et al.*, 2003). NO-suppressed Arabidopsis mutants (*Atnoa1* and *Atnia1nia2noa1-2*) generally exhibit a pale phenotype (Guo and Crawford, 2005; Lozano-Juste and Leon, 2010) and have relatively low chlorophyll and carotenoid concentrations (Tewari *et al.*, 2019). These mutant plants, however, do show enhanced greening in their leaves when they are grown in the presence of sucrose (Van Ree *et al.*, 2011), uranium, and on Murashige and Skoog growth medium (Tewari *et al.*, 2019). Under these treatment regimes, these allegedly NO-suppressed mutants have been shown to produce NO (Tewari *et al.*, 2019). Hence, the concomitant greening in the foliage of NO-suppressed plants in conditions where NO is induced suggests an involvement of NO in chlorophyll biosynthesis. This could, for example, be accomplished by increasing the functional Fe status. An interconversion between different redox forms of Fe depending on the Fe

and NO status of the plant cells might be the core metabolic process that drives Fe homeostasis in the plant.

NO does not affect the synthesis of phytosiderophores

Strategy II plants, such as monocot grasses, secrete metal-chelating phytosiderophores [(mugineic acid (MA) and 2-deoxymugineic acid (DMA)] from their roots into the rhizosphere for Fe acquisition (Wirén *et al.*, 1994). The non-proteinogenic amino acid nicotianamine (NA) serves as an intermediate in the biosynthetic pathway of MAs (Wirén *et al.*, 1994; Nozoye *et al.*, 2013). Phytosiderophores are synthesized from *S*-adenosyl methionine. To enable the uptake of insoluble Fe^{3+}, roots secrete DMA into the root zone through TOM1 (transporter of MA family phytosiderophores1) to chelate Fe^{3+}. In this way, phytosiderophores enable Fe^{3+} uptake without its prior reduction to Fe^{2+}. Uptake of the Fe^{3+}–phytosiderophore complexes into the root symplast is facilitated by the activity of the transporters Yellow Stripe (YS) and Yellow Stripe-Like (YSL) (Kobayashi *et al.*, 2005; Kobayashi and Nishizawa, 2014). Under Fe-deficient conditions, strategy II plants often show enhanced secretion of MA and DMA, and the expression of genes related to NA synthase (*NAS1, NAS2*), NA aminotransferase (*NAAT*), and Fe uptake and transport [*iron regulated transporter 1* (*IRT1*), *ferric reductase oxidase 1* (*FRO1*), *YS1*, *YSL2*, and *YSL15*] (Kobayashi *et al.*, 2005; Nozoye *et al.*, 2013; Wang *et al.*, 2015). The regulation of transcript levels of *NAS1*, *NAS2*, *YS1*, *YSL2*, and *YSL15* by NO treatment in Fe-deficient plants is largely unknown. However, the transcription of *LeIRT1* and *LeFRO1* has been reported to be up-regulated by the application of the NO donor GSNO (Graziano and Lamattina, 2007). On the other hand, application of the NO scavenger carboxy-PTIO (cPTIO) to Fe-deficient wheat plants did not affect phytosiderophore release into the nutrient solution or the expression of *TaNAAT* in roots (Garnica *et al.*, 2018). Jasmonic acid-induced NO generation in *Taxus* cells (Wang and Wu, 2005) and jasmonic acid biosynthesis-related genes have been reported to be up-regulated by Fe deficiency, along with genes involved in Fe uptake and transport, namely *TOM1*, *YSL15*, *YSL2*, *NRAMP1* (*natural resistance-associated macrophage protein 1*), *NRAMP5*, and *IRT1* (Kobayashi *et al.*, 2016).

NO affects Fe-related transcription factors

The genes involved in Fe^{3+}–DMA complex uptake, Fe^{2+} uptake, and Fe translocation have been reported to be differentially regulated by various transcription factors (TFs) (Kobayashi *et al.*, 2014). In rice, DMA-mediated Fe uptake is regulated by the basic helix-loop-helix (bHLH) TFs, iron-related TF 2 and 3 (OsIRO2 and OsIRO3), and iron deficiency-responsive element binding factor 1 (OsIDEF1) (Kobayashi *et al.*, 2014; Kobayashi and Nishizawa, 2014). OsIDEF1 and OsIRO3 also regulate the expression of *OsIRT1*. OsIDEF1, an important transcriptional regulator of Fe deficiency, is proposed to act as an Fe sensor (Kobayashi *et al.*, 2014; Kobayashi, 2019). TFs belonging to the subgroup Ib bHLHs, including rice OsIRO2 and Arabidopsis AtbHLH38, AtbHLH39, AtbHLH100, and AtbHLH101, induce the expression of large subsets of Fe-deficiency-inducible genes for Fe uptake and translocation (Kobayashi *et al.*, 2005; Kobayashi, 2019). The subgroup IVb bHLH TFs (rice OsIRO3 and Arabidopsis POPEYE), on the other hand, repress the expression of Fe-deficiency-inducible Fe homeostasis genes, including subgroup Ib bHLHs (Zheng *et al.*, 2010; Kobayashi, 2019). TFs such as Fer-like Fe deficiency-induced TF (FIT) and IDEF1 interact with proteins involved in signaling pathways of phytohormones, oxidative stress, and excess metals. Thus, FIT and IDEF1 have been proposed to act as hubs for the integration of environmental stimuli to modulate Fe-deficiency responses (Kobayashi, 2019). The responses of IDEF1 and IRO3 to NO are largely unknown, whereas FIT has been shown to be positively regulated by NO (Meiser *et al.*, 2011; Hindt and Guerinot, 2012; Kobayashi, 2019). Moreover, NO inhibits the degradation of FIT (Kobayashi, 2019). Finally, the TF-encoding genes *AtbHLH38*, *AtbHLH39*, and *AtMYB72* are strongly induced by Fe deficiency and GSNO in Arabidopsis (Huang *et al.*, 2018). Orthologs of these TF-encoding genes showed marked changes after NO treatment, suggesting that NO-responsive genes and pathways might be regulated by these TFs (Huang *et al.*, 2018). The data obtained so far clearly indicate a potential interaction between NO, TFs, and the regulation of Fe uptake during Fe deficiency in plants, but further research is needed to fully clarify the regulatory pathways.

NO affects the synthesis and activity of various Fe-responsive proteins

NADPH oxidase

Plant NADPH oxidases are known as respiratory burst oxidase homologues (RBOHs). RBOHs catalyze the production of superoxide anions ($O_2^{\cdot-}$) and are involved in several essential processes in plants (Marino *et al.*, 2012). The NADPH oxidase is a membrane-integrated glycoprotein with an apparent molecular mass of ~91 kDa (Sumimoto, 2008). A typical NADPH oxidase consists of a cytoplasmic C-terminal homologous to ferredoxin:NADPH oxidoreductase and an N-terminal region containing two hemes with a structure similar to that of cytochrome *b* (Sumimoto, 2008; Tewari *et al.*, 2014).

It has frequently been observed that NADPH oxidase-like activity is enhanced by the addition of NO-donating compounds such as SNP or SNAP (Tewari *et al.*, 2008a). Suppression of NADPH oxidase activity in the presence of the NO scavenger PTIO further suggests the involvement of NO

in its regulation (Tewari *et al.*, 2008a). Since NADPH oxidases contain a heme center as a structural component, it can be hypothesized that NO enhances NADPH oxidase-like activity by increasing Fe availability for heme biosynthesis. Most NADPH oxidases produce $O_2^{\cdot -}$ towards the plant apoplasm, which may mediate the reduction of external (apoplastic) Fe(III) to Fe(II) under Fe-deficient conditions. Fe(III) reduction *in vivo* may be aided by $O_2^{\cdot -}$ formation (Brüggemann *et al.*, 1993). Thus, increased accumulation of $O_2^{\cdot -}$ in Fe-deficient plants may promote Fe acquisition from the apoplast (Tewari *et al.*, 2005; Tewari, 2019). Moreover, the Arabidopsis Rho-like GTPase 6 mutant (*rop6*), which produces considerably less ROS under Fe-deficient conditions, probably due to down-regulation of *RBOHF* and *RBOHD*, synthesizes less chlorophyll in the foliage despite a higher expression of *FRO2* and *IRT1* and the accumulation of sufficient Fe in the roots (Zhai *et al.*, 2018). Another gene encoding a small GTPase, *OsRab6a*, in rice has been shown to be involved in Fe acquisition. Overexpression of *OsRab6a* resulted in higher expression levels of genes encoding NA synthases (*NAS1*, *NAS2*), which are involved in the synthesis of MA-family phytosiderophores, IRT1, and IRO2 in Fe-deficient rice plants (Yang and Zhang, 2016). IRO2 is a bHLH TF involved in the regulation of genes that are responsive to Fe deficiency (Yang and Zhang, 2016). Since small GTPases regulate both NO and RBOH-mediated $O_2^{\cdot -}$ production (Selvakumar *et al.*, 2008), it appears that $O_2^{\cdot -}$ probably participates in increasing functional Fe availability in Fe-deficient plants (Fig. 4).

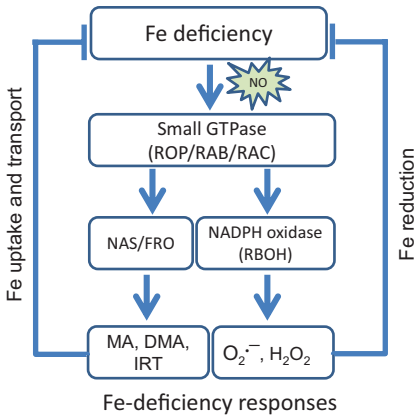

Fig. 4. Schematic representation showing Fe-deficiency-induced NO generation and activation of small GTPases (ROP/RAB/RAC). Small GTPases may activate nicotianamine synthase (NAS), NADPH oxidase (RBOH), and ferric chelate reductase (FRO) to regulate the Fe status of the cell through the synthesis of nicotianamine, mugineic acid (MA), 2-deoxymugineic acid (DMA), and IRT. FRO and RBOH, using NADH and NADPH, may reduce Fe^{3+} to Fe^{2+} through the formation of superoxide anion ($O_2^{\cdot -}$). $O_2^{\cdot -}$ and its dismutation product, H_2O_2, may consequently activate different Fe-deficiency responses in plants.

Ferric reductase oxidase

In soils, Fe mainly exists as ferric oxide (Fe_2O_3). The reduction of Fe_2O_3 is essential for Fe bioavailability (Yi and Guerinot, 1996; Guerinot, 2001). In plants subjected to Fe deficiency, the active growing parts of roots often exhibit a strong increase in Fe^{3+}-chelate reductase (FCR) activity (Yi and Guerinot, 1996). This enzyme belongs to the FRO family and is a metalloprotein (Robinson *et al.*, 1999; Jain *et al.*, 2014). *FRO2* shows sequence similarity with yeast ferric reductase, *FRE1*, and a subunit of the human NADPH oxidase, gp91phox, *RBOH*. FRO2 belongs to a superfamily of flavocytochromes (Mukherjee *et al.*, 2006; Jain *et al.*, 2014). FRO2 reduces Fe^{3+}-chelates at the root–rhizosphere interface (Yi and Guerinot, 1996; Guerinot, 2007). After reduction, Fe^{2+} is transported across the cell membrane of the root epidermis by the Fe^{2+} transporter IRT1 (Kobayashi and Nishizawa, 2014). The expression of *FRO* and *IRT* genes is regulated by a FER/FER-like TF (Yuan *et al.*, 2005; Buet *et al.*, 2019).

Like NADPH oxidase, FCR is up-regulated by both Fe deficiency and NO supply in tomato (Graziano and Lamattina, 2007) and peanut (Song *et al.*, 2016; Song *et al.*, 2017). The genes involved in Fe uptake, *LeFRO1* and *LeIRT1*, are expressed in a NO-dependent manner in tomato plants, as their expression was found to be suppressed in the presence of the NO scavenger cPTIO (Jin *et al.*, 2009). Moreover, the up-regulation of FCR activity by GSNO was found to be abolished in the presence of cPTIO (Graziano and Lamattina, 2007). Fe deficiency also enhanced H^+-ATPase activity along with FCR activity, which respectively causes acidification of the apoplasm and facilitates the reduction of Fe^{3+}. Hence, NO may act as a signaling molecule and activate FCR through a FIT-mediated transcriptional regulation of *FRO2* (Chen *et al.*, 2010). NO acts as a stabilizing factor for FIT protein abundance in Arabidopsis (Meiser *et al.*, 2011). FIT, a bHLH TF, may interact with other TFs, such as EIN3/EIL1, which augment FIT stability (Lingam *et al.*, 2011) and increase Fe acquisition in plants (Meiser *et al.*, 2011). Using GSH and a GSNO inhibitory compound, R7 (3-amino-N-(3-methylphenyl)thieno[2,3-b]pyridine-2-carboxamide), it has been found that NO acts upstream of both the FIT and Ib bHLH transcriptional networks (Kailasam *et al.*, 2018). In peanut, the activities of both H^+-ATPase and FCR were found to be higher in NO-supplied plants than Fe-supplied plants (Song *et al.*, 2016; Song *et al.*, 2017); it therefore appears that both H^+-ATPase and FCR are regulated by NO via transcriptional regulation of FIT. Fe^{2+} reduced by FCR is internalized into the cell by IRT1 (Barberon *et al.*, 2014). Fe-deficient tomato and peanut plants generally accumulate more NO in their roots, which suggests that NO indeed plays a role in the enhanced availability of functional Fe in plants.

Heme oxygenase

Heme oxygenase (HO), a monomeric protein, is a ubiquitous enzyme that catalyzes the cleavage of heme to biliverdin with the release of Fe and carbon monoxide (CO) (Shekhawat and Verma, 2010; Li *et al.*, 2013) (Fig. 5). HO1 is highly conserved in

invertebrates, higher plants, algae, and bacteria (Wei *et al.*, 2011; Li *et al.*, 2013). In plants, HO is involved in Fe acquisition/mobilization, probably due to heme degradation (Shekhawat and Verma, 2010). HO has been reported to be induced by NO and to cause lateral root formation in rice (Chen *et al.*, 2012; Huei Kao, 2014). Fe deficiency induced the expression of *AtHO1* at the transcriptional and translational levels. Overexpression of AtHO1 could confer plant tolerance to Fe deficiency by increasing the expression of *AtFIT*, *AtFRO2*, and *AtIRT1*, the activity of FCR, and Fe accumulation (Li *et al.*, 2013). FCR activity was found to be up-regulated in the presence of the NO donor SNP and abolished in the presence of the NO scavenger cPTIO both in wild-type Arabidopsis plants and in the *AtHO1* knockdown mutant. *AtHO1* overexpression results in the generation of more NO, whereas knockdown of *AtHO1* results in decreased NO production in plants (Li *et al.*, 2013). Thus, AtHO1 is involved in Fe homeostasis (Fig. 5A). However the HO null mutant *athy1* (*hypocotyl1*) produces more NO, which results in the inhibition of root elongation due to excess Cd^{2+} (Han *et al.*, 2014). *athy1* exhibits severe Fe-deficiency effects (up-regulation of *FRO2* and *IRT1*) despite the accumulation of NO under Cd stress (Han *et al.*, 2014) (Fig 5B). The accumulation of heavy metals generally induces Fe-deficiency-like symptoms in plants (Tewari *et al.*, 2002; Kumar *et al.*, 2008).

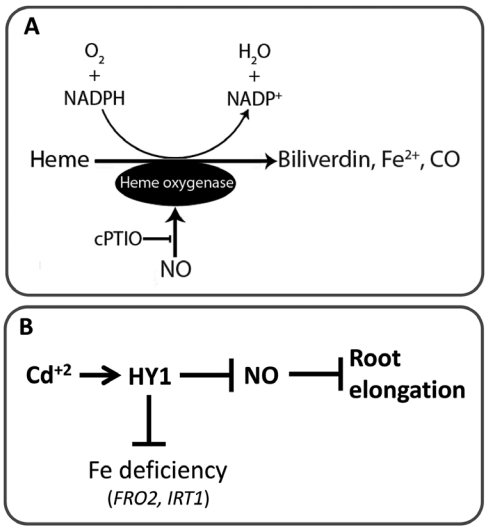

Fig. 5. Schematic representation showing the heme oxygenase-catalyzed cleavage of heme to biliverdin with the release of Fe^{2+} and carbon monoxide (CO). (A) Heme oxygenase is up-regulated in the presence of NO, which results in increased Fe^{2+} availability for metabolic function. (B) The heme oxygenase-defective mutant *hy1* exhibits severe Cd^{2+}-induced Fe-deficiency responses due to the production of NO. Overexpression of heme oxygenase (HY1) results in inhibition of Fe-deficiency responses (suppressed expression of *FRO* and *IRT1*) due to low NO production and inhibition of root growth in Arabidopsis plants. Redrawn after Han *et al.* (2014) Arabidopsis HY1 confers cadmium tolerance by decreasing nitric oxide production and improving iron homeostasis. Molecular Plant 7, 388–403. Copyright (2014), with permission from Elsevier.

Nitrate reductase

NR is a Mo-containing enzyme. Arabidopsis expresses two isoforms of NR, NIA1 and NIA2. NIA2 exhibits higher NR activity, which supports the idea that the different NR isoforms have specific functions in plants (Mohn *et al.*, 2019). NR catalyzes the following reaction:

$$NO_3 + NADH + H^+ \rightarrow NO_2 + NAD^+ + H_2O$$

The C-terminal domain of NR carries a flavin adenine dinucleotide cofactor that accepts two electrons from NADH or NADPH and passes them on to the *b5*-cytochrome heme in the middle domain. NIA1 is an efficient NiR and produces NO by catalyzing the following reaction under hypoxic conditions (León and Costa-Broseta, 2019):

$$NO_2 + e + H^+ \rightarrow NO + H_2O$$

NR has been proposed to be one of the enzymatic sources of NO in plants. Fe deficiency decreased the activity of NR and NiR in the cyanobacterium *Agmenellum quadruplicatum* (Hardie *et al.*, 1983) and in cucumber (Borlotti *et al.*, 2012), and the expression of their genes in barley (Higuchi *et al.*, 2011). Plasma membrane-bound NOFNiR is a heme-Fe-containing enzyme that catalyzes the reduction of nitrite to NO (Tejada-Jimenez *et al.*, 2019). NR-mediated and mitochondrial electron transport chain-dependent reductions of nitrite to NO are proposed to be the most relevant sources of NO production in higher plants (León and Costa-Broseta, 2019). NO production under Fe-deficient conditions is expected to be low via the ferredoxin–NR pathway. A study that applied the NR inhibitor tungstate showed NR-mediated production of NO in the roots of Fe-deficient tomato plants (Graziano and Lamattina, 2007). However, a suppressed transcript level of genes encoding NR has been found in leaves of Fe-deficient barley (Higuchi *et al.*, 2011) and cucumber (Borlotti *et al.*, 2012). Despite the fact that both NR and NiR are dependent on Fe for their functioning, Fe-deficient plants often produce more NO (Chen *et al.*, 2010; Graziano and Lamattina, 2007), which suggests the possible involvement of an additional pathway in NO generation in plants.

Cytochrome c oxidase

Cytochromes are Fe-containing proteins that are found in chloroplasts and mitochondria. Fe-deficient conditions are often associated with decreased cytochrome levels (Vigani et al., 2009, 2013). However, cytochrome c oxidase (COX), a major mitochondrial site of NO production in hypoxic plant cells (Igamberdiev *et al.*, 2014), did not change significantly in Fe-deficient conditions (Vigani *et al.*, 2009). COX is also an important target of NO. NO inhibits COX activity by competitively binding to the heme Fe^{2+} group at the O_2-binding

site (Brown, 2001; Igamberdiev *et al.*, 2014). The fact that Fe deficiency does not have a significant effect on COX levels or activity may enable NO levels to stay sufficiently high in Fe-deficient plants and hence also help to increase Fe availability.

Lipoxygenase

Lipoxygenases are a family of (non-heme) Fe-containing enzymes. Lipoxygenases catalyze the dioxygenation of polyunsaturated fatty acids containing a *cis*,*cis*-1,4- pentadiene (Dufrusine *et al.*, 2019). The N-terminal domain confers Ca^{2+}-dependent membrane-binding ability. The larger catalytic C-terminal α-helical domain harbors the non-heme catalytic Fe (Porta and Rocha-Sosa, 2002). The expression of the lipoxygenase-encoding gene *LOX2* was found to be enhanced in Arabidopsis plants treated with gaseous NO (Huang *et al.*, 2004). The activity of lipoxygenase was found to be up-regulated by methyl jasmonate in a NO-dependent manner in *Taxus chinensis* cell suspension cultures treated with methyl jasmonate (Wang and Wu, 2005). The lipoxygenase activity of these cells was also enhanced by the application of the NO donor SNP, and abolished by the NO scavenger PTIO (Wang and Wu, 2005). It appears that NO generated in *T. chinensis* cell suspension cultures enhanced the availability of functional Fe, which was concomitantly utilized in the synthesis of lipoxygenase and thus led to the increased activity of this Fe-containing enzyme.

Aconitase

Aconitase is a Fe-regulatory Fe–S protein (Brouquisse *et al.*, 1986). Fe is the metal component of the prosthetic group that is required for the enzyme's stability and activity, and the Fe cluster is responsible for the spatial orientation of the substrates (citrate and isocitrate). Aconitase catalyzes the isomerization of citrate to isocitrate in the tricarboxylic acid cycle (Igamberdiev *et al.*, 2014). Poor availability of Fe results in decreased aconitase activity (Vigani *et al.*, 2013) and the accumulation of organic acids (citrate and malate) in plants (López-Millán *et al.*, 2001). The production of organic acids may have the potential to increase Fe availability in plants for metabolic function (López-Millán *et al.*, 2001; Borlotti *et al.*, 2012). Fe-deficient plants generally produce copious amounts of $O_2^{\cdot -}$, which may inhibit aconitase activity, as the Fe–S cluster of this enzyme is sensitive to oxidation by $O_2^{\cdot -}$. Decreased aconitase activity in response to the exogenous application of the NO producers 6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanimine (NOC-9) or 3-morpholinosydnonimine-HCl (SIN-1) (Poborilova *et al.*, 2013) suggests that NO-induced generation of $O_2^{\cdot -}$ by activating NADPH oxidase activity (Tewari *et al.*, 2008c) is responsible for the inhibition of aconitase. The inhibition of aconitase results in reduced electron flow through the mitochondrial electron transport chain and a subsequent decrease in ROS and NO production (Durner and Klessig, 1999; Wendehenne *et al.*, 2004; Gupta *et al.*, 2012; Igamberdiev *et al.*, 2014).

NO modulates the activities of antioxidant enzymes

Superoxide dismutases

Superoxide dismutases (SODs) represent the first line of defense against oxidative stress, as they dismutate $O_2^{\cdot -}$ into H_2O_2 (Alscher *et al.*, 1997; Tewari *et al.*, 2004; Allen *et al.*, 2007; Tewari *et al.*, 2013a). SODs are polyphyletic metalloproteins. The following SOD forms, with different metal components, have been reported: Fe-SODs, Mn-SODs, Cu/Zn-SODs, and Ni-SODs (Wolfe-Simon *et al.*, 2006; Broadley *et al.*, 2012). To protect themselves against deleterious $O_2^{\cdot -}$, Fe-deficient plants often exhibit increased SOD activity (Tewari *et al.*, 2005; Sun *et al.*, 2007; Tewari *et al.*, 2013a; Tewari, 2019). Under Fe-deficient conditions, a compensatory mechanism appears to function by overproducing other SOD forms, such as Mn-SOD and Cu/Zn-SODs, in *Chlamydomonas* (Allen *et al.*, 2007). NO suppresses the increased SOD activity following Fe deficiency. This observation supports the involvement of NO in improving functional Fe status probably by raising $O_2^{\cdot -}$ production (Sun *et al.*, 2007; Kumar *et al.*, 2010; Tewari *et al.*, 2019). The resulting decrease in the dismutation of $O_2^{\cdot -}$ to H_2O_2 presumably explains the decrease in H_2O_2 concentration observed in Fe-deficient maize plants treated with the NO producer SNP (Kumar *et al.*, 2010; Tewari, 2019), while the extra $O_2^{\cdot -}$ can be expected to promote the reduction of Fe^{3+} to Fe^{2+} in the apoplast (Graziano *et al.*, 2002; Tewari, 2019). Excessive build-up of $O_2^{\cdot -}$ may be avoided by NO itself scavenging some $O_2^{\cdot -}$ to form peroxynitrite ($ONOO^-$), which readily becomes protonated and finally decomposes to H^+ and NO_3^- (Beligni and Lamattina, 2001; Tewari *et al.*, 2013b). Although $ONOO^-$ is a strong oxidant that can be highly cytotoxic and mediates apoptosis in animal cells, it is relatively non-toxic in plants (Delledonne, 2005; Valderrama *et al.*, 2007).

Catalases

Catalases (CATs) are highly active heme-Fe-containing enzymes that do not require cellular reductants as they primarily catalyze the dismutation of two molecules of H_2O_2 to water and O_2 (Mhamdi *et al.*, 2010). There are two types of CATs: 'typical' or 'monofunctional' heme-based tetrameric CATs and bifunctional catalase–peroxidases that are structurally distinct proteins (Mhamdi *et al.*, 2010). Bifunctional catalase–peroxidases are similar to the heme-containing peroxidases such as ascorbate peroxidase (APX) and fungal cytochrome c peroxidase (Mhamdi *et al.*, 2010). The activity of CAT has been reported to be decreased by Fe deficiency and generally recovered on exogenous application of the NO-producing

compound SNP (Sun *et al.*, 2007; Kumar *et al.*, 2010; Song *et al.*, 2017). This could help to avoid the build-up of damaging concentrations of H_2O_2. The NO-accumulating rice mutant *nitric oxide excess1* (*noe1*), which is defective in CAT activity, has been shown to accumulate H_2O_2, resulting in increased leaf cell death. These observations suggest that NO is an important mediator of H_2O_2-induced leaf cell death in the absence of OsCATC (Lin *et al.*, 2012). Up-regulation of CAT by exogenous application of NO reflects improved functional Fe status with alleviation of Fe-deficiency symptoms. Since CATs constitutively contain heme-Fe, several researchers have considered CAT activity as an index of Fe nutritional status (Mehrotra and Gupta, 1990; Iturbe-Ormaetxe *et al.*, 1995; Tewari *et al.*, 2005; Broadley *et al.*, 2012).

Peroxidases

Peroxidases (hydrogen donor: H_2O_2 oxidoreductase) belong to a large multigene family and participate in a range of biological processes, namely lignin and suberin formation, cross-linking of cell wall components, as well as ROS and reactive nitrogen species metabolism and the hypersensitive response during pathogen infections (Almagro *et al.*, 2008). Plant peroxidases are heme-containing enzymes that catalyze the single one-electron oxidation of several substrates at the expense of H_2O_2 ($2RH+H_2O_2 \rightarrow 2R\cdot+2H_2O$). Plant peroxidases, which belong mainly to the class III peroxidases, are Ca^{2+}-glycoproteins and are located in vacuoles and cell walls (Barceló *et al.*, 2007). Peroxidase, with heme-Fe as cofactor, also showed an expected response similar to other heme-Fe enzymes and could, like CATs, be considered as an index of the functional Fe status of plants (Tewari *et al.*, 2005; Tewari, 2019). The activity of peroxidase is increased by NO supply using application of the NO producer SNP, and also showed a significant positive correlation with chloroplastic pigments (Kumar *et al.*, 2010; Tewari, 2019). Sun *et al.* (2007) also observed a similar increase in the activities of heme-Fe-containing enzymes upon treating Fe-deficient plants with SNP.

Ascorbate peroxidases

Plant APXs are also heme-containing enzymes (Correa-Aragunde *et al.*, 2015). They belong to the class I peroxidases, which, in contrast to the class III peroxidases, are not glycoproteins, and are located in chloroplasts, mitochondria, peroxisomes, and the cytosol (Almagro *et al.*, 2008). APX reduces H_2O_2 to H_2O with ascorbate as an electron donor [$2ASC+H_2O_2 \rightarrow 2$ monodehydroascorbate (MDA)$+2H_2O$] (Correa-Aragunde *et al.*, 2015). APXs, along with the enzymes of the ascorbate–glutathione (ASC–GSH) cycle, are crucial in the control of cellular redox status in plants (Foyer, 2018). APX activity is down-regulated under Fe deficiency (Iturbe-Ormaetxe *et al.*, 1995; Tewari *et al.*, 2005) and enhanced under excess Fe supply (Kampfenkel *et al.*, 1995) to control the damage caused by ROS. APX expression is reported to be regulated by Fe availability in the tissues (Ishikawa *et al.*, 2003; Fourcroy *et al.*, 2004). Exogenous NO application up-regulates APX activity (Sun *et al.*, 2007; Tewari *et al.*, 2019), and APX activity is closely correlated with increases in the chlorophyll concentration (Kumar *et al.*, 2010). This is quite understandable, as both heme and chlorophyll possess a tetrapyrrole ring as a structural constituent and have been shown to share a common pathway, which is affected by Fe availability.

Glutathione reductase

Glutathione reductase (GR) is a ubiquitous flavoprotein oxidoreductase found in both eukaryotes and prokaryotes that converts oxidized glutathione (GSSG) to the reduced form (GSH), using NADPH as an electron donor ($GSSG+NADPH+H^+ \rightarrow 2GSH+NADP^+$) (Bashir *et al.*, 2007). In plants, GR is the GSH-regenerating enzyme of the ASC–GSH cycle (Romero-Puertas *et al.*, 2006). The activity of GR has been reported to be enhanced under Fe-deficient conditions (Iturbe-Ormaetxe *et al.*, 1995; Bashir *et al.*, 2007; Sun *et al.*, 2007). Transcripts of *GR1* and *GR2* were also up-regulated under Fe-deficient conditions (Bashir *et al.*, 2007). It appears that GR plays a key role in removing ROS under Fe-deficient conditions in cooperation with glutathione peroxidase and SODs (Bashir *et al.*, 2007). GR may also be involved in Fe homeostasis by regenerating GSH. GSH protects Fe-deficient plants against detrimental effects of Fe deficiency, including Fe chlorosis (Ramírez *et al.*, 2013; Gheshlaghi et al., 2019, 2020). An increased level of GSH has been reported in Fe-deficient sugar beet roots (Zaharieva and Abadía, 2003; Zaharieva *et al.*, 2004). Exogenous supply of the NO producer SNP down-regulates GR activity (Sun *et al.*, 2007) as NO increases Fe availability (Fig. 6).

NO-mediated post-translational regulation of proteins

Nitration of tyrosine residues and *S*-nitrosylation of cysteine residues are common post-translational modifications (PTMs) that modulate the function of target proteins (Astier and Lindermayr, 2012; Gupta *et al.*, 2020a). Protein nitration mediated by $ONOO^-$ adds a nitro group ($-NO_2$) to the tyrosine residue (Ortega-Galisteo *et al.*, 2012). *S*-nitrosylation is mediated by GSNO, which results in the binding of a NO group to a cysteine residue of a protein. PTMs can change the activity and function of proteins (Astier and Lindermayr, 2012; Begara-Morales *et al.*, 2018). Histone deacetylases appear to be targets of *S*-nitrosation or *S*-glutathionylation, resulting in the hyperacetylation of specific regions of chromatin (genes) (Ageeva-Kieferle *et al.*, 2019). The NO-responsive TF WRKY, which is involved in stress tolerance, has a cysteine or a tyrosine

residue near to the WRKY domain, suggesting a mechanism of regulation by *S*-nitrosation or tyrosine nitration (Mengel *et al.*, 2017; Buet *et al.*, 2019). WRKY46 has been reported to regulate translocation of Fe from root to shoot under Fe-deficient conditions (Yan *et al.*, 2016). In addition, GSNO, resulting from the *S*-nitrosylation of glutathione, specifically mediates the Fe-starvation signal to the central modulator FIT, which is involved in Fe acquisition and utilization (Kailasam *et al.*, 2018).

$ONOO^-$-mediated nitration of Mn-SOD, Fe-SOD, and CuZn-SOD results in the inactivation of SOD activity (Begara-Morales *et al.*, 2016). Enzymes of the ASC–GSH cycle are also modulated through PTM by NO (Begara-Morales *et al.*, 2016; Corpas *et al.*, 2019). APX activity has been reported to be reduced by tyrosine nitration (Begara-Morales *et al.*, 2014), whereas *S*-nitrosylation results in enhanced APX activity (Correa-Aragunde *et al.*, 2013). Increased levels of the phytohormone auxin cause APX1 denitrosylation and partial inhibition of APX1 activity in Arabidopsis roots (Correa-Aragunde *et al.*, 2013). During programmed cell death, however, *S*-nitrosylation of cytosolic APX leads to its ubiquitination and degradation (de Pinto *et al.*, 2013). Likewise, both tyrosine nitration and *S*-nitrosylation have been reported to inhibit the activity of monodehydroascorbate reductase (Begara-Morales *et al.*, 2015). GR activity, however, is not significantly affected by these NO-related PTMs (Begara-Morales *et al.*, 2015). This behavior suggests that APX and GR try to detoxify H_2O_2 and maintain the regeneration of GSH, respectively, and consequently maintain the redox status of the cell under nitro-oxidative cellular conditions (Begara-Morales *et al.*, 2015).

NO forms complexes with Fe

NO contains an unpaired electron but remains uncharged. Because of its free radical nature, it can adopt an energetically more favorable electronic configuration by gaining or losing an electron. In this way NO can exist as three interchangeable species: the NO radical (NO·), the nitrosonium cation (NO^+), and the nitroxyl radical (NO^-).

$$NO^+ \underset{-e^-}{\overset{+e^-}{\rightleftharpoons}} NO^{\cdot} \underset{-e^-}{\overset{+e^-}{\rightleftharpoons}} NO^-$$

NO· is isoelectronic with the dioxygen monocation (O_2^+), and NO^+ is isoelectronic with CO and CN^-, while NO^- is isoelectronic with O_2, having a triplet ground state. Bonding between a NO^- ligand and a metal accounts for the formation of metal-nitrosyl (M-NO) complexes, which have structural and electronic analogies with biological oxygen activators (Lewandowska, 2013).

NO^+ has been isolated as a series of stable salts, and is a useful synthetic and oxidizing agent. NO^+ is a key species in the process of nitrosation, in which the NO^+ group is transferred from a carrier compound to a nucleophilic center (Ramirez *et al.*, 2011). An example of such a molecule is GSNO (Ramirez *et al.*, 2011). GSNO has been considered to be a major reservoir of NO and a long-distance signaling molecule (Begara-Morales *et al.*, 2018; Corpas and Palma, 2018). NO^+ has in all likelihood an extremely short independent life span in biological media, although metal complexes may function as transport agents. NO can bind to metals to form M-NO complexes [mononitrosyl iron complex (MNIC) and dinitrosyl iron complex (DNIC)]. The presence of both MNICs and DNICs has been observed in sorghum, soybean, and wheat embryos exposed to various NO donors (SNP, DETA NONOate, and GSNO) (Buet and Simontacchi, 2015). DNICs are relatively more stable than NO or free Fe (Graziano and Lamattina, 2005). Thus, these M-NO species are known to play a key role in biological NO storage and transport (Li and Li, 2016). M-NO complexes formed under neutral physiological conditions may act as links between the different redox states of NO (Begara-Morales *et al.*, 2013). In conclusion, the formation of Fe-NO

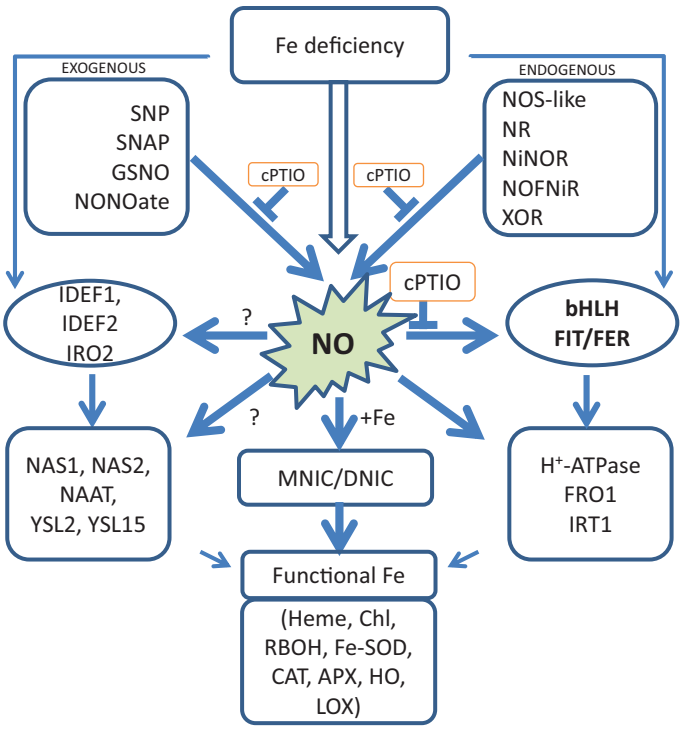

Fig. 6. Schematic representation of NO-mediated Fe homeostasis in plants. Exogenous supply or endogenous generation of NO results in the expression of FIT/FER, bHLH transcription factors that activate the expression of H^+-ATPase, FRO, and IRT1 involved in acidification, reduction of Fe^{3+}-chelate, and Fe uptake in strategy I plants. cPTIO is a NO scavenger. IDEF1, IDEF2, and IRO2 probably participate in the expression of NAAT and YSL in strategy II plants. Question marks indicate probable regulation of IDEF1, IDEF2, and IRO2 by NO, which has not yet been elucidated. NO may increase Fe availability by forming mono-/dinitrosyl iron complexes (MNIC/DNIC). MNIC/DNIC may participate in the delivery of functional Fe to target tissues/cells. These mechanisms increase functionally active Fe, which participates in the synthesis of heme and chlorophyll (Chl), and the activation of Fe-related proteins such as RBOH, Fe-SOD, catalase (CAT), ascorbate peroxidase (APX), heme oxygenase (HO), and lipoxygenase (LPX).

complexes might be a strategy for storing and stabilizing free NO and Fe within a living system (Lewandowska *et al.*, 2011; Hsiao *et al.*, 2019). These complexes may travel relatively long distances and may release Fe and NO at target sites.

NO is associated with Fe-toxicity-mediated root inhibition

Various studies have established that NO is a key player in Fe homeostasis by regulating the availability of Fe for metabolic processes (Murgia *et al.*, 2002; Graziano and Lamattina, 2005, 2007). The root tip is the organ that can sense excess Fe and Fe toxicity, and it plays a direct role in the modulation of the root system architecture (Zhang *et al.*, 2019). The activity of the root tip meristem of Arabidopsis plants has been found to be arrested by excess Fe, which results in massive NO accumulation (Zhang *et al.*, 2019). Ethylene can antagonize excess-Fe-inhibited root growth by controlling the NO status of the root tip (Zhang *et al.*, 2019). GSNO reductase (GSNOR) maintains root meristem activity by inhibiting Fe-dependent nitrosative and oxidative cytotoxicity (Ramirez *et al.*, 2011; Li *et al.*, 2019). GSNOR has also been reported to be required for root tolerance to excess Fe. This also opens an opportunity to address crop production under excess Fe conditions using GSNOR variants (Li *et al.*, 2019).

NO in ferritin modulation

Ferritin is the cellular storehouse of Fe. An overload of Fe in the tissues can lead to oxidative damage due to the formation of highly deleterious OH· in the Fenton reaction (Halliwell, 1999, 2006). The structures of plant and animal ferritin are very similar; they are formed of 24 subunits arranged to form a hollow sphere in which ~2000–4500 Fe atoms can be stored. Ferritin is able to sequester Fe in a non-toxic but bioavailable form (Briat *et al.*, 2009). The storage and buffering of Fe in dedicated compartments, such as the apoplast and different organelles (vacuole, plastids, and mitochondria), protects the cell from the potential damaging effects of Fe excess (Briat *et al.*, 2007; Briat *et al.*, 2009). The loss of ferritin genes in the *fer1,3,4* triple mutant of Arabidopsis leads to a massive accumulation of Fe in the apoplastic space, and the chloroplasts of these plants apparently have no more buffering capacity for Fe (Roschzttardtz *et al.*, 2013). Thus, the Arabidopsis *fer1,2,3* mutant activates Fe efflux and/or represses Fe influx to limit the amount of Fe in the cell (Roschzttardtz *et al.*, 2013). Ferritin transcripts have been reported to accumulate quickly upon Fe exposure, which consequently translates into the accumulation of ferritin protein (Ramirez *et al.*, 2011). NO also accumulates in the plastids after Fe exposure, and it appears to act downstream of Fe and upstream of a PP2A-type serine/threonine phosphatase to promote an increase in the expression of *AtFer1* (Arnaud *et al.*, 2006). NO is required for Fe-induced ferritin synthesis, as treatment with the NO scavenger cPTIO prevents the accumulation of ferritin transcripts in Arabidopsis cell suspensions exposed to excess Fe (Murgia et al., 2002, 2004). The expression of *AtFer1* has been shown to be repressed under Fe-limiting conditions. The involvement of a *cis*-acting Fe-dependent regulatory sequence (IDRS) within the *AtFer1* promoter sequence has been reported (Arnaud *et al.*, 2006). The repressor may bind to the IDRS and prevent the transcription of *AtFer1* under Fe-limiting conditions. This repressor is ubiquitinated upon the restoration of Fe availability and subsequently degraded through a 26S proteasome-dependent pathway, leading to the transcription of *AtFer1* (Arnaud *et al.*, 2006). Fe appears not to be necessary for NO-mediated accumulation of ferritin transcripts, since the presence of a NO donor itself is able to induce the accumulation of ferritin transcripts in Arabidopsis cell suspensions pre-treated with the Fe chelating agents deferoxamine or ferrozine. Thus, NO seemingly acts downstream of Fe in the induction of ferritin transcripts, and appears to be a key signaling molecule for the regulation of Fe homeostasis in plants (Murgia *et al.*, 2002).

Conclusions and future perspectives

On the basis of various studies on NO-mediated improvement of Fe status, it is concluded that NO is indeed involved in maintaining Fe homeostasis of plants. Significant progress has been made in the past decade in understanding the mechanisms of Fe homeostasis and its interaction with NO in plants. For example, NO has been shown to increase internal Fe availability. However, further research is needed to fully understand the NO-mediated regulation of various TFs, transcripts, enzymes, and transporters, including those involved in the biosynthesis of MA-family phytosiderophores. Fe-deficiency-induced basal NO generation appears to be insufficient and thus external sources of NO are needed to meet the levels of NO required to increase Fe availability. Supplying NO-producing chemicals to plants could be used as a potential way to reduce Fe deficiency, including lime-induced chlorosis, in growing plants and crops.

Various antioxidant enzymes (SOD, APX, and MDAR) are affected by NO-mediated PTMs such as tyrosine nitration and *S*-nitrosylation. However, how these NO-mediated PTMs affect various Fe-responsive enzymes/proteins under either Fe deficiency or Fe excess is largely unknown. Moreover, different downstream targets of NO-mediated signaling events leading to increased functional Fe availability in plants have not yet been fully explored. In addition, NO-mediated signaling events culminating in the modulation of antioxidant defenses through the targeted activation/deactivation of specific TFs via Ca^{2+}/calmodulin kinase and/or MAPK cascades have not yet been adequately addressed.

Acknowledgements

RKT is grateful to Department of Science and Technology-Science and Engineering Research Board (DST-SERB) New Delhi, for a Teachers Associateship for Research Excellence (TAR/2019/000064).

Author contributions

RKT conceived the idea and wrote the original draft of the manuscript. NH reviewed and edited the text. NH and MW provided some pictures of NO localization in the plant organs/cells and helped in the preparation of the manuscript. All authors read and approved the manuscript.

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