Nitric oxide and glutathione impact the expression of iron uptake- and iron transport-related genes as well as the content of metals in *A. thaliana* plants grown under iron deficiency

Emmanuel Koen,[1,3] Katarzyna Szymańska,[2] Agnès Klinguer,[1] Grażyna Dobrowolska,[2] Angélique Besson-Bard[1] and David Wendehenne[1,*]

[1]Université de Bourgogne; UMR 1347 Agroécologie; Pôle Mécanisme et Gestion des Interactions Plantes-microorganisms; Dijon, France; [2]Institute of Biochemistry and Biophysics; Polish Academy of Sciences; Warsaw, Poland; [3]AgroParisTech; ENGREF; Paris, France

Mounting evidence indicate that nitric oxide (NO) acts as a signaling molecule mediating iron deficiency responses through the upregulation of the expression of iron uptake-related genes. Accordingly, NO donors such as nitrosoglutathione (GSNO) were reported to improve the fitness of plants grown under iron deficiency. Here, we showed that glutathione, a by-product of GSNO, triggered the upregulation of the expression of iron uptake- and transport-related gene and an increase of iron concentration in *Arabidopsis thaliana* seedlings facing iron deficiency. Furthermore, we provided evidence that under iron deficiency, NO released by GSNO did not improve the root iron concentration but impacted the content of copper. Collectively, our data highlight the complexity of interpreting data based on the use of NO donors when investigating the role of NO in iron homeostasis.

Introduction

Nitric oxide (NO) is a diatomic gas that has been shown to function as a cell-signaling molecule with diverse physiological roles in plants. Indeed, NO is involved in developmental processes, hormonal signaling and adaptive response to environmental stresses.[1] The mechanisms by which it exerts its effects are being discovered and include the mobilization of classical second messengers such as Ca^{2+} and cyclic GMP, the regulation of protein kinase activities and gene expression, the modulation of lipid signaling and the post-translational modification of numerous proteins.[1-5]

During the past 10 y, it has been recognized that NO also acts as a signaling molecule mediating iron deficiency responses.[6] Treatment with artificially-released NO was shown to improve the fitness of maize and tomato plants grown under iron deficiency.[7,8] Under such conditions, the NO-treated plants displayed increased root hair development, higher chlorophyll contents and reduced interveinal chlorosis typical of iron deficiency. Further supporting these findings, NO was reported to be rapidly produced in roots of plants exposed to iron deficiency.[8] Once

produced, it initiates an iron-starvation pathway promoting the expression of genes which products are involved in iron uptake. In *Arabidopsis thaliana*, these proteins include the major ferrous transporter IRT1 (iron-regulated transporter 1), the root plasma membrane ferric reductase FRO2 (ferric reductase oxidase 2) and the basic helix-loop-helix transcription factor FIT (fer-like Fe deficiency-induced transcription factor) which positively regulates *IRT1* and *FRO2* expression. Accordingly, treatment of plant roots with NO donors such as nitrosoglutathione (GSNO) was reported to promote the expression of these genes or their orthologs in other plant species.[8] In addition, these genes were shown to be induced by NO endogenously produced in roots of *A. thaliana* seedlings in response to the iron deficiency caused by cadmium treatment.[9] The list of genes positively regulated by NO under iron deficiency also includes *NAS4* (Nicotianamine Synthase 4).[9] The corresponding protein catalyzes the synthesis of nicotianamine, a non proteinaceous amino-acid involved in iron transport and distribution in planta.[10]

Interestingly, although NO treatments of plants such as maize or rice were able to counteract the iron deficiency symptoms, no increase in iron content in plant organs was observed.[7]
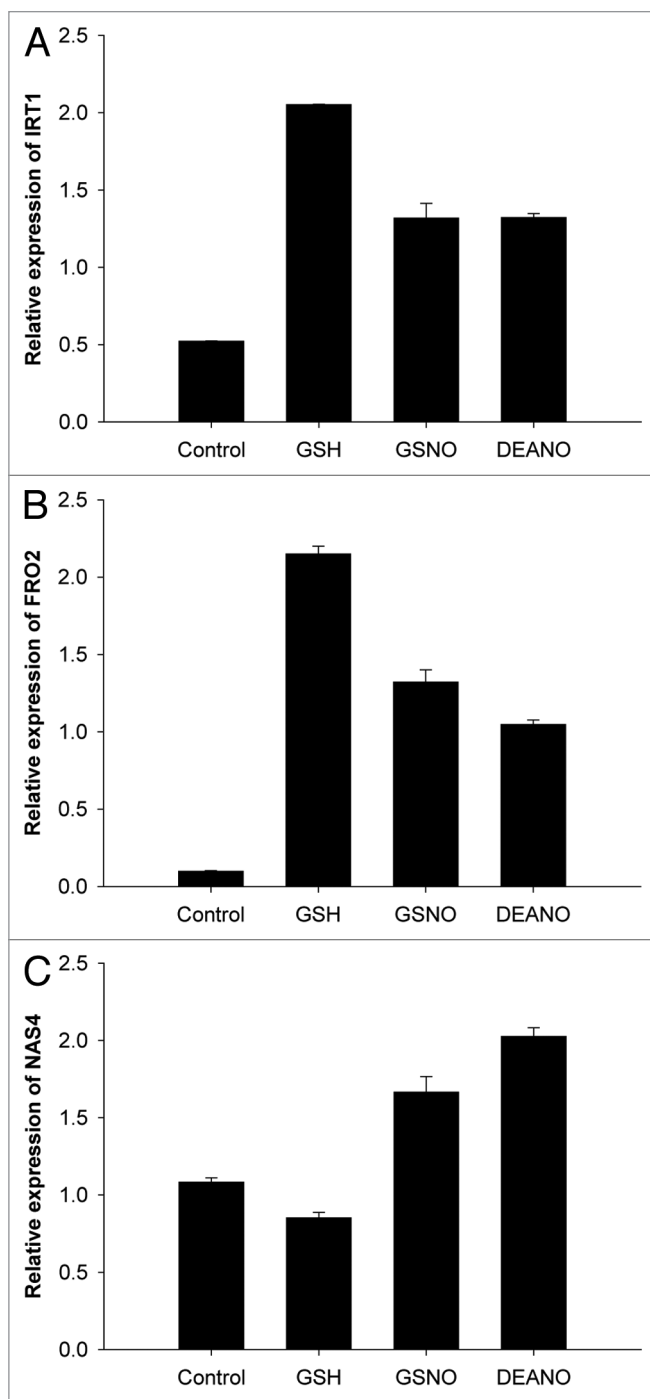
Figure 1. Incidence of GSNO, DEANO and GSH on the accumulation of *IRT1*, *FRO2* and *NAS4* transcripts in roots of plants exposed to iron deficiency. (A) *IRT1* transcript accumulation, (B) *FRO2* transcript accumulation, (C) *AtNAS4* transcript accumulation in roots. Plants were cultivated 4 weeks in iron-sufficient conditions (Fe 50 μM) and then transferred 48 h in iron deficient (Fe 5 μM) conditions. Then, plants were treated with GSNO (50 μM), GSH (50 μM) or DEANO (50 μM) for 24 h. Transcript accumulation of *IRT1*, *FRO2* and *NAS4* in roots were measured by qRT-PCR. Data are expressed as mean normalized expression relative to the appropriate housekeeping gene *AtYLS8* (arbitrary units) and are the means of triplicates (± SE).

It has been proposed that exogenously applied NO does not improve iron uptake by roots but, instead, favors an increase in iron availability for instance through the formation of dinitrosyl iron complexes.[11,12] These complexes, formed by the interaction between NO and ferric iron with thiols-containing ligands, could transport and deliver iron from roots to other plant organs. Furthermore, the beneficial effects of NO could be related to its ability to prevent plants from the oxidative stress caused by iron deficiency.[13]

To further investigate the role of NO in the plant adaptive response to iron deficiency, in the present investigation we analyzed the incidence of exogenously applied NO in iron, zinc, copper and manganese accumulations in roots of *A. thaliana* plants exposed to iron deficiency.

Results

A. thaliana seedlings grown for 4 weeks in hydroponic conditions with 50 μM of iron were transferred for 96 h in the same medium in which the concentration of iron was reduced to 5 μM. Forty eight hours after the transfer, plant roots were exposed to 50 μM of the NO donors GSNO or diethylamine-NONOate (DEANO). Then, after 48 h of incubation with the NO donors, the root metal concentrations were examined by induced coupled plasma-optical emission spectrometry (ICP-OES). To check whether the NO donor treatments impact the expression of iron-uptake/transport genes in our conditions, the accumulation of *IRT1*, *FRO2* and *NAS4* in roots were analyzed 24 h after the supplementation of the medium with these compounds.

Compared with the control plants, GSNO triggered a 2.5 and 13-fold increase of *IRT1* and *FRO2* transcript accumulation, respectively (**Fig. 1A and B**). These data fit well with those of Graziano and Lamattina[8] who reported that GSNO mediates the expression of the orthologs of the *A. thaliana IRT1* and *FRO2* genes in tomato plants under iron deprivation. Because GSNO releases both glutathione (GSH) and NO, the putative effect of GSH was also assessed (**Fig. 1A and B**). Surprisingly, GSH induced the strongest upregulation of *IRT1* and *FRO2* expression. Indeed, compared with GSNO treatment, the levels of *IRT1* and *FRO2* mRNA were increased by 1.6 fold. To confirm that the effect of GSH was at least partly caused by NO released by the donor, plants were also exposed to the NO donor DEANO. Whereas diethylamine alone did not impact the accumulation of the transcripts of interest (data not shown), DEANO promoted the expression of both genes by 2.6 and 11.5 fold as compared with control plants, respectively. Collectively, these data indicate that NO as well as GSH treatments amplify the accumulation of *IRT1* and *FRO2* transcripts in roots of *A. thaliana* seedlings grown in iron deficient condition. Interestingly, different data were obtained regarding *NAS4* transcripts (**Fig. 1C**). Indeed, whereas a 1.5 and 1.8 fold increase of *NAS4* mRNA accumulation occurred in response to GSNO and DEANO, respectively, GSH treatment slightly reduced their levels. Therefore, the regulation of the expression of *NAS4* might differ from those of *IRT1* and *FRO2*.

Concerning the concentration of iron, no differences were observed between control plants and plants exposed to GSNO or DEANO (**Fig. 2A**). In contrast, compared with WT plants, iron concentration was significantly increased by 1.3-fold in response to GSH. Regarding copper (**Fig. 2B**), while its concentration was unchanged in GSH- and DEANO-treated plants compared with control plants, differences were observed in response to GSNO treatment. Indeed, compared with WT plants, the concentration of copper was significantly decreased by 25%. In the case of zinc and manganese, no significant differences were observed between control plants and plants exposed to GSH, GSNO or DEANO (**Fig. 2C and D**). Taken together, these data indicate that GSNO impacted the concentration of copper. However, both NO donors did not improve the iron concentration of plants grown under iron deficiency, thus confirming Graziano et al.[7] previous observation in maize. Furthermore, GSH itself affected the concentration of iron.

Discussion

The present investigation confirmed previous data showing that NO released by NO donors promotes the expression of *IRT1* and *FRO2* in plants suffering from iron deficiency without favoring an increase of iron uptake. It also confirms our recent study highlighting a role for NO in the regulation of the expression of NAS4.[9] The observation that GSH, a by-product of GSNO, also influenced the plant response to iron deficiency raises the question of the role of GSH in iron deficiency as well as its interplay with NO. It has been previously reported that iron deficiency enhances the level of GSH.[14] According to Ramirez et al.,[11] GSH might act as a thiol-containing ligand in the formation of dinitrosyl iron complexes and, consequently, might contribute to the increase of iron availability in plant tissues. Furthermore, it seems plausible that the feeding of plants with GSH might increase the intracellular pool of GSNO which constitutes a stable and mobile endogenous reservoir of NO. The rise in GSNO content could promote the NO-dependent mechanisms contributing to the iron deficiency responses such as the upregulation of *IRT1* and *FRO2* transcript expression. Besides these aspects, our work also highlights the importance of GSH as a control when assessing the effects of GSNO on plant biological processes.

Others important findings of the present investigation concern the influence of the NO donors on the accumulation of other metals. Indeed, our data indicate that GSNO also impacted the concentration of copper. This observation is not surprising as the ion homeostasis networks in plants seem closely linked.[15] Notably, iron, manganese and zinc are all taken up by IRT1 and FRO2 is also able to reduce Cu.[16] Therefore, copper, iron, zinc and manganese might affect each other's uptake.

Finally, our data showed that the plant response to NO might differ according to the NO donor. Indeed, GSNO and not DEANO triggered a decrease in the concentration of copper. Beside the fact that GSNO releases both NO and GSH, GSNO mainly releases nitrosonium cation (NO^+) while DEANO releases NO in its radical form ($NO^\bullet$). Furthermore, the kinetic of NO release differs between both NO donors. Indeed, the release of NO from DEANO occurs within seconds in aqueous solution[17] whereas the release form GSNO is slower.[18] Although speculative, we assume that these parameters might influence the action of NO. Here too, this discussion highlights the complexity of interpreting data based on the use of NO donors.

Materials and Methods

Plant material and growth conditions. Experiments were performed using *A. thaliana* Columbia ecotype (Col-0) wild-type. Seeds were surface sterilized by immersion in a solution containing 1.2% bleach and 50% EtOH for 5 min and placed for 3 d at 4°C in 0.15% agar. Then, they were sown in seed holders filled with 0.75% agar and put in a cover placed above the nutrient solution container (Araponics). The medium (0.25 mM $Ca(NO_3)_2$, 0.5 mM KNO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 50 μM H_3BO_3, 19 μM $MnCl_2$, 10 μM $ZnCl_2$, 1 μM $CuSO_4$, 0.02 μM Na_2MoO_4, 50 μM Fe-Na-EDTA) was changed once a week. Plants were allowed to grow for 4 weeks in the following conditions: 10 h day (200 μE.m^{-2}.s^{-1} light intensity, 20°C) / 14 h night (18°C) with 70% relative humidity. After 4 weeks of culture, roots were washed 3 times with the nutritive solution containing 5 μM Fe-EDTA instead of 50 μM. Then, plants were grown for 96 h on the hydroponic medium with 5 μM Fe- EDTA.

Plant treatments. Forty eight hours after their transfer into iron-deficient conditions, plants were treated with 50 μM of GSNO, GSH or DEANO. GSNO and GSH were dissolved in water, DEANO was prepared as previously described.[17] As a control for DEANO effects, plants were treated with 50 μM diethylamine prepared in water.

Determination of metal concentration. Roots were collected 48 h after the addition of the NO donors or GSH into the culture medium. The concentration of metals was determined in the roots by induced coupled plasma-optical emission spectrometry (ICP-OES) as previously reported.[9]

qRT-PCR analyses. Root tissues were collected after the treatments and immediately frozen in liquid nitrogen before total RNA extraction using Trizol reagent according to the manufacturer's instructions (Invitrogen). A DNase (Promega) treatment was performed on 5 μg of total RNA to prevent genomic DNA contamination. RNA samples were subsequently used for reverse transcription (ImpromII™ Reverse Transcriptase kit, Promega) with anchored oligo(dT15) (Promega) and 0.4 mM deoxynucleotide triphosphates. The resulting cDNAs were subjected to a 2-fold dilution with water and one μl of each cDNA sample was assayed by qPCR (7900HT, Applied Biosystems®). Expression levels were calculated relatively to the housekeeping gene *AtYLS8* (*At5g08290*) using the relative standard curve method. For each sample, target quantity of the gene of interest was determined by interpolating the value from the standard curve made from a cDNA pool which enables to take into consideration the efficiency of amplification. The value was then divided by the target quantity of the housekeeping gene.

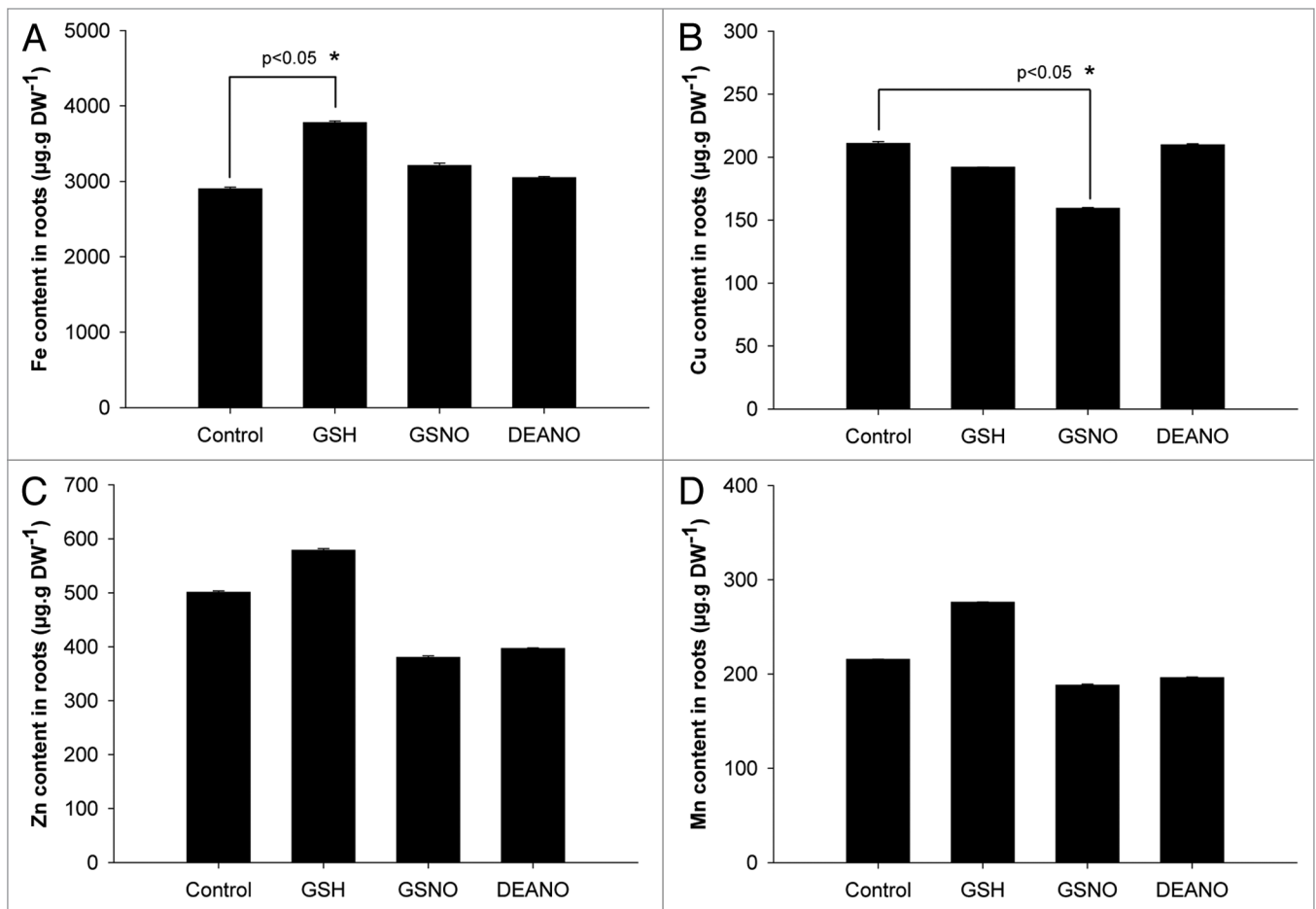Primer sequences were as follows: *AtNAS4*-fw: 5'-AGA AGG TTA AGG TGG TCG AGC-3', *AtNAS4*-rev:

Figure 2. Impact of GSNO, DEANO and GSH on the concentrations of iron, manganese, zinc and copper in roots of plants exposed to iron deficiency. (A) Iron concentration, (B) copper concentration, (C) zinc concentration, (D) manganese concentration. Plants were cultivated 4 weeks in iron-sufficient conditions (Fe 50 μM) and then transferred 48 h in iron deficient (Fe 5 μM) conditions. Then, plants were treated with GSNO (50 μM), GSH (50 μM) or DEANO (50 μM) for 48 h. The concentration of metals was determined in the roots by ICP-OES. Each value represents the means ± SD of nine measurements (three replicates per experiment performed three times). Stars represent significant differences from control according to ANOVA on ranks followed by a comparison vs. control (p < 0.05).

5'-ACAGTTACACGCGAGATCCGA-3'; *IRT1*-fw: 5'-CGG TTG GAC TTC TAA ATG C-3', *IRT1*-rev: 5'-CGA TAA TCG ACA TTC CAC CG-3'; *FRO2*-fw: 5'-CGA TCG TTT CCT TCG GTT TC-3', *FRO2*-rev: 5'-AAT CCG AGC AGC GAG CAA-3'; *AtYLS8*-fw: 5'-GTC ATT TAT CTG GTG GAC ATC ACT-3', *AtYLS8*-rev: 5'-CCG AAA CAG TAA GCA AAA AC-3'.

Chemicals. All chemicals were purchased from Sigma-Aldrich.

Statistical analysis. In order to detect significant differences between the different treatments, ANOVA on ranks were performed with the statistical program SigmaPlot release 11.0 (Systat Software) followed by a comparison vs. control with the Dunn's Method (*p < 0.05).

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