The Plant Journal (2002) **30**(5), 521–528

Nitric oxide mediates iron-induced ferritin accumulation in *Arabidopsis*

Irene Murgia[1,*], Massimo Delledonne[2] and Carlo Soave[1]

[1]*Sezione di Fisiologia e Biochimica delle Piante, Dipartimento di Biologia, Università degli Studi di Milano, via Celoria 26, 20133 Milano, Italy, and*

[2]*Dipartimento Scientifico e Tecnologico, Università degli Studi di Verona, Strada Le Grazie 15 – Ca' Vignal – 37134 Verona – Italy*

Received 15 February 2002; accepted 27 February 2002.
[*]For correspondence (fax +39 02 50314815; e-mail irene.murgia@unimi.it).

Summary

Nitric oxide (NO) is a signaling molecule that plays a critical role in the activation of innate immune and inflammatory responses in animals. During the last few years, NO has also been detected in several plant species and the increasing number of reports on its function in plants have implicated NO as an important effector of growth, development and defense. Analogously to animals, NO has been recently shown to inhibit tobacco aconitase. This suggests that NO may elevate free iron levels in the cells by converting tobacco cytoplasmic aconitase into a mRNA binding protein that negatively regulates accumulation of ferritin.

We investigated the possible role of NO as a regulator of ferritin levels in *Arabidopsis* and found that the NO-donor sodium nitroprusside (SNP) induces accumulation of ferritin both at mRNA and protein level. Iron is not necessary for this NO-mediated ferritin transcript accumulation, since SNP is still able to induce the accumulation of ferritin transcript in *Arabidopsis* suspension cultures pre-treated with the iron chelants DFO or ferrozine. However, NO is required for iron-induced ferritin accumulation, as the NO scavenger CPTIO prevents ferritin transcript accumulation in *Arabidopsis* suspension cultures treated with iron. The pathway is ser/thr phosphatase-dependent and necessitates protein synthesis; furthermore, NO mediates ferritin regulation through the IDRS sequence of the *Atfer1* promoter responsible for transcriptional repression under low iron supply.

NO, by acting downstream of iron in the induction of ferritin transcript accumulation is therefore a key signaling molecule for regulation of iron homeostasis in plants.

Keywords: ferritin, nitric oxide, iron, oxidative stress, IDRS sequence, *Arabidopsis*.

Introduction

Iron may bind to heme groups, iron-sulfur clusters, or directly associate with proteins and is essential for many cellular functions. Nonetheless, in the free ionic form iron is toxic as it can catalyze the formation of reactive oxygen species (ROS) through the Haber-Weiss reaction. These ROS in turn damage cell membranes, DNA, and proteins (Bowler *et al.*, 1992; Guerinot and Yi, 1994; Noctor and Foyer, 1998).

Iron homeostasis is strongly dependent on ferritins, which are iron-storage proteins present in bacteria (Andrews *et al.*, 1991), plant (Lobreaux *et al.*, 1992b; Prouhdon *et al.*, 1996) and animal cells (Harrison and Arosio, 1996). Plant and animal ferritins have very similar structures and are formed by 24 subunits arranged to form a protein coat able to sequester up to 4500 iron atoms in a non-noxious form (Harrison and Arosio, 1996; Theil, 1987). In leaves, ferritin is located in chloroplasts and is developmentally regulated, being more abundant in developing and senescent leaves (Briat and Lobreaux, 1997). Ferritin accumulation is induced by an excess of iron (Lobreaux *et al.*, 1992a), as well as by abscissic acid (Fobis-Loisy *et al.*, 1995; Lobreaux *et al.*, 1993), photoinhibition and ozone (Murgia *et al.*, 2001). All of these factors either elicit or mimic oxidative stress. In maize cell cultures, accumulation of ferritin may occur without iron addition when induced by H_2O_2 (Savino *et al.*, 1997). Antioxidants, how-

ever, suppress iron-induced ferritin accumulation (Savino *et al.*, 1997) and ascorbic acid, which is a redox sensor in chloroplasts, modulates the capacity of ferritin to take up or release iron (Laulhere and Briat, 1993).

Despite the growing number of physiological inducers of plant ferritin discovered to date, little is known about the regulatory molecules acting downstream of iron. In animals, ferritins are regulated post-transcriptionally. Ferritin mRNA contain iron-responsive elements (IREs) in their 5′ untranslated region that function as binding sites for two related transacting factors, namely the iron regulatory proteins IRP1 and IRP2. When bound to the IRE in the ferritin transcript, the IRP inhibits translation of the transcript (Hentze and Kühn, 1996). IRP1 is a bifunctional protein; when iron is abundant it possesses a [4Fe-4S] cluster and it acts as cytoplasmic aconitase; when iron levels are low, its [4Fe-4S] cluster disassembles and the apoprotein acquires IRP activity, thus repressing ferritin translation (Hentze and Kühn, 1996). High levels of iron cause the [4Fe-4S] cluster to reconstitute and the protein regains aconitase activity. As such, the two activities are mutually exclusive.

In mammals, nitric oxide (NO) controls many diverse functions including iron homeostasis (Durner *et al.*, 1999; Nathan, 1995; Schmidt and Walter, 1994; Stamler *et al.*, 1997). NO induces the aconitase to IRP1 transition, causing an increase of the labile iron pool (LIP), through a reduction of the ferritin pool (Cairo and Pietrangelo, 2000; Gardner *et al.*, 1997; Hentze and Kühn, 1996). Recent reports, however, show specific down-regulation of IRP2 by an oxidized form of NO, the nitrosonium ion NO^+ (Kim and Ponka, 1999; Kim and Ponka, 2000), which causes a reduction of the LIP through translationally controlled induction of ferritin synthesis (Recalcati *et al.*, 1998).

In plants, NO has been shown to have a role in the hypersensitive response and is involved in programmed cell death (Delledonne *et al.*, 2002a). It also induces various defense genes, including pathogenesis-related proteins and enzymes of phenylpropanoid metabolism that are involved in the production of lignin and the secondary signal salicylic acid (Delledonne *et al.*, 1998; Delledonne *et al.*, 2001; Delledonne *et al.*, 2002b; Durner *et al.*, 1998; Hausladen and Stamler, 1998). Furthermore, NO is involved in the adaptive plant responses against drought stress (Mata and Lamattina, 2001) as well as in growth and development (Beligni and Lamattina, 2000; Leshem and Pinchasov, 2000; Leshem *et al.*, 1998).

Recently, NO has been found to inactivate tobacco aconitase (Navarre *et al.*, 2000). Plant and mammalian cytoplasmic aconitases possess a high degree of homology, especially in those regions responsible for IRP activity (Navarre *et al.*, 2000; Peyret *et al.*, 1995). This evidence therefore suggests that, with respect to aconitase-dependent ferritin regulation, NO may have similar roles in both plants and vertebrates (Klessig *et al.*, 2000). It has been proposed that NO might down-regulate ferritin through the action of the aconitase/IRP-1 plant homolog. Such a homolog would thus elevate free iron levels, cause an increase in the concentration of ROS through the Fenton reaction and provide a defensive function against pathogen attack (Klessig *et al.*, 2000; Wendehenne *et al.*, 2001).

When considering this hypothesis, two characteristic aspects of plant ferritins must be taken into account. First, they are regulated mainly at a transcriptional level, although post-transcriptional regulation has been observed in the maize mutant *ys1* (Fobis-Loisy *et al.*, 1996). Second, in plants the ferritin genes do not contain a canonical IRE sequence. Only very recently, a common scheme of iron regulation of ferritin expression by iron in plant and animal cells has emerged, although the targets appear to be different, namely DNA in plants and mRNA in animals. The promoters of the ferritin genes in soybean, maize and *Arabidopsis* indeed contain sequences responsible for transcriptional repression under low iron supply, which are referred to as the FRE (soybean) and IDRS sequences (maize and *Arabidopsis*) (Petit *et al.*, 2001; Wei and Theil, 2000). The mechanism of iron-dependent regulation of plant ferritin also appears to be based on gene derepression in analogy to the IRP/IRE mRNA derepression seen in animals. However, the factors acting in the animal and plant pathways are likely to be different, since animal IREs share no homology with either plant FRE or IDRS sequences.

We have characterized the function of NO in the mediation of iron homeostasis in plant cells by studying its involvement in the regulation of ferritin mRNA and protein. It was found that NO stimulates accumulation of both ferritin mRNA and protein. Our results indicate that NO acts downstream of iron, through the IDRS sequence, in the signaling cascade leading to ferritin accumulation.

Results

The NO donor SNP induces accumulation of ferritin transcript and protein in Arabidopsis leaves

Sodium nitroprusside (SNP), which releases NO^+ *in vivo* (Bogdan, 2001; Delledonne *et al.*, 1998; Kim and Ponka, 2000) was infiltrated into *Arabidopsis* leaves. Northern blot analysis revealed that 1 mM SNP induced accumulation of ferritin transcript at 1 h after infiltration (Figure 1a). Elevated levels of ferritin transcripts remained up to 24 h and declined thereafter. Neither water nor the SNP analog potassium ferricyanide, which is unable to release NO, had any effect on ferritin accumulation (Figure 1a). The weak accumulation of ferritin transcripts observed at later time

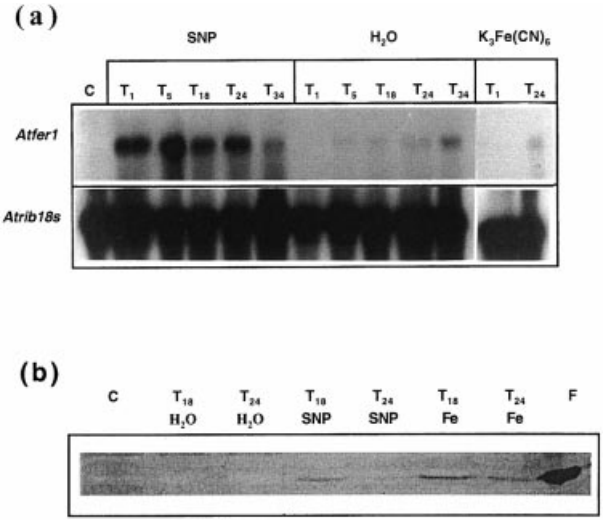

Figure 1. Ferritin accumulation in *Arabidopsis* leaves infiltrated with the NO-donor SNP.
(a) Northern blot analysis of *Arabidopsis* leaves infiltrated with 1 mM SNP, water, or 1 mM $K_3Fe(CN)_6$ in the dark. T_1, T_5, T_{18}, T_{24}, T_{34} indicates samples collected after 1, 5, 18, 24, 34 h of infiltration, respectively. C represents the control (leaves collected before infiltration). Five μg total RNA was loaded in each lane. Filters were hybridized either with the ferritin *Atfer1* probe (upper panel) or the constitutively expressed *Atrib18s* probe (lower panel).
(b) Western blot analysis of *Arabidopsis* leaves infiltrated with 1 mM SNP, 500 μM Fe-citrate, or water, in the dark. T_{18} and T_{24} refer to samples collected after 18 and 24 h infiltration. C represents the negative control (leaves collected before infiltration). The positive control F consists of 10 μg protein crude extract from pea seeds, rich in ferritin. Twenty-five μg protein crude extracts were loaded in all the other lanes. A polyclonal antibody against pea ferritin was used as primary antibody.

points after treatment with water or potassium ferricyanide is most likely due to the mechanical stress of the infiltration process itself. Western blot analysis on protein crude extracts from infiltrated leaves was performed using a polyclonal antibody raised against pea ferritin (Van Wuytswinkel *et al.*, 1999). These experiments showed that upon infiltration of 1 mM SNP, accumulation of the ferritin protein was detectable between 18 and 24 h (Figure 1b) and, as expected, it was not detectable at earlier time-points (data not shown). A similar time delay between mRNA and protein accumulation has been previously observed in leaves that have been overloaded with iron (Savino *et al.*, 1997). By comparing the relative increases of ferritin mRNA and protein, it appeared that protein accumulation was less pronounced with respect to mRNA levels. Similar results have also been observed when ferritin accumulation was induced by either abscissic acid (ABA) (Lobreaux *et al.*, 1993) or photoinhibition (Murgia *et al.*, 2001). The accumulation of protein upon infiltration with 500 μM Fe-citrate was also not particularly prominent (Figure 1b). However, in either case, the signal strength may be underestimated as the primary polyclonal antibody is against pea ferritin.

NO mediates iron-induced ferritin accumulation

Stimulation of ferritin transcript accumulation was also observed in *Arabidopsis* suspension cultures. As expected, Fe-citrate and H_2O_2 were found to be strong and weak inducers, respectively (Figure 2a). SNP concentrations as low as 0.15 mM induced accumulation of ferritin mRNA within 3 h (Figure 2a). As shown in Figure 2(b), this response could be essentially prevented by the addition of the NO scavenger CPTIO (carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3 oxide) (Delledonne *et al.*, 1998).

To determine whether the iron-induced accumulation of ferritin transcripts is mediated by NO, *Arabidopsis* suspension cultures were incubated with iron in the presence of increasing amounts of CPTIO: 100 μM CPTIO drastically reduced and 1 mM CPTIO completely abolished the iron-induced accumulation of ferritin mRNA (Figure 2b). This result suggests that NO acts downstream of iron in the signaling cascade. To further reinforce this hypothesis, *Arabidopsis* suspension cultures were iron-depleted for 20 h with 500 μM deferoxamine mesylate (DFO), a strong iron chelating agent and then treated with either 1 mM or 5 mM SNP. In these conditions, the addition of the NO donor caused the accumulation of ferritin transcript to the same extent as in control cells that had not been treated with DFO and therefore were not starved for iron (Figure 2c). A similar result was obtained using 1 mM ferrozine as iron chelating agent (not shown).

NO acts on the IDRS sequence, a cis-regulatory element of the Atfer1 ferritin promoter

It has recently been demonstrated that the promoter of *Arabidopsis* ferritin *Atfer1* possesses an iron dependent regulatory sequence (IDRS) that is responsible for repression of gene transcription under low iron supply; this result was achieved by testing *Arabidopsis* plants transformed with an *Atfer1* promoter-Gus gene fusion in which 1400 bp of promoter *Atfer1* sequence has a mutated (At1400m*IDRS line) or wt (At1400IDRS line) IDRS (Petit *et al.*, 2001). In At1400m*IDRS lines, GUS activity is constitutively high, indicating that mutation of the IDRS sequence causes gene derepression. On the contrary, GUS activity could be increased in At1400IDRS lines after iron treatment (Petit *et al.*, 2001).

The two *Arabidopsis* transformed lines were infiltrated with either 500 μM Fe-citrate or 1 mM SNP. As expected, iron derepression factors (ratio between the GUS activities from treated to untreated plants) were those expected: 1.2 for At1400 m*IDRS line and 12 for the At1400IDRS line (Figure 3). Most notably, with respect to stimulation of GUS activity, SNP functions in the same way as iron, being the SNP derepression factor 1.4 in the At1400 m*IDRS line

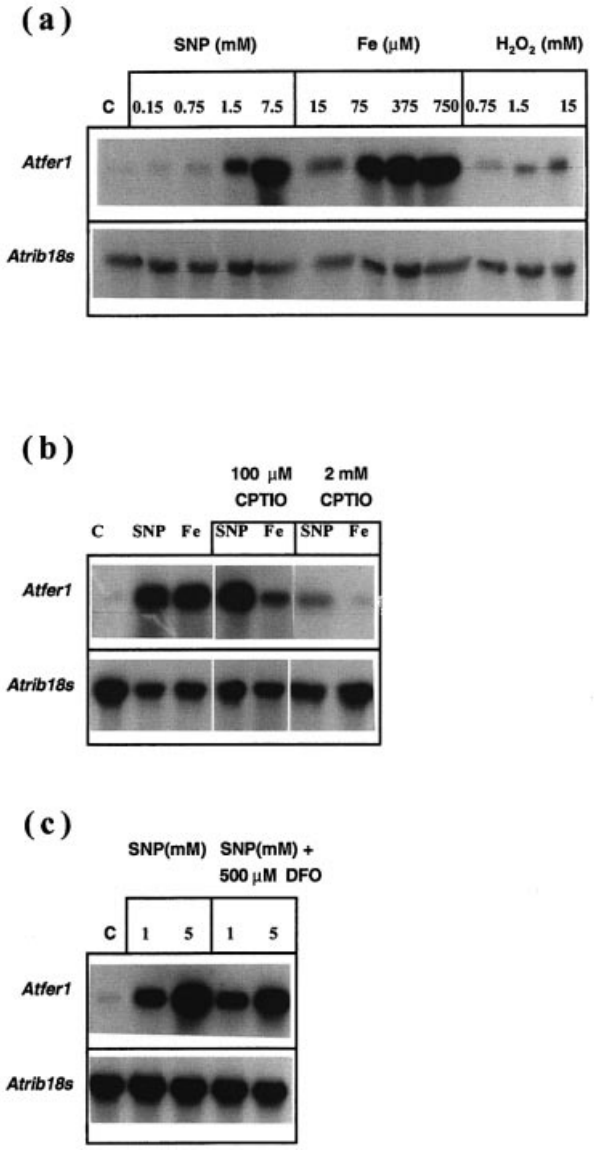

Figure 2. NO acts downstream of iron in the pathway leading to ferritin transcript accumulation.
(a) Northern blot analysis of *Arabidopsis* suspension cultured cells, 3 h after treatment with increasing concentrations of SNP, Fe-citrate or H_2O_2. C represents the control (untreated cells). Five μg total RNA was loaded in each lane. Filters were hybridized either with the ferritin *Atfer1* probe (upper panel) or the constitutively expressed *Atrib18s* probe (lower panel).
(b) Northern blot analysis of *Arabidopsis* suspension cultures, 3 h after treatment with 5 mM SNP or 100 μM Fe-citrate, in the absence or presence of either 100 μM or 2 mM CPTIO. C represents the control (untreated cells).
(c) Northern blot analysis of *Arabidopsis* suspension cultured cells, 3 h after treatment with 1 mM or 5 mM SNP, with or without pre-treatment with 500 μM DFO for 20 h. C represents the control (untreated cells). DFO alone had no effect on ferritin transcript accumulation (not shown).

and 11 in the At1400IDRS line (Figure 3). This result indicates that NO mediates ferritin regulation through the IDRS sequence.

Ser/thr phosphatase activity and protein synthesis are required for SNP-induced ferritin transcript accumulation

Reversible protein phosphorylation/dephosphorylation events play numerous roles in mediating intracellular signaling processes (Barford, 1996). In plants, dephosphorylation of phosphoserine or phosphothreonine by ser/thr phosphatases is involved in many diverse cellular functions (Smith and Walker, 1996), such as auxin transport (Garbers *et al.*, 1996) and metabolism (Carter *et al.*, 1990).

A calyculine-sensitive protein phosphatase activity is also involved in the regulation of *Zmfer1* ferritin gene in maize cells, an activity required for iron-induced expression of the gene (Savino *et al.*, 1997). To investigate whether the NO-mediated accumulation of ferritin depends on phosphorylation/dephosphorylation events, *Arabidopsis* suspension cultures were pre-treated with calyculin prior to the addition of iron or SNP. In this case, calyculin prevented both iron- and SNP-induced transcript accumulation, indicating that a ser/thr phosphatase is also required for NO-induced accumulation of ferritin mRNA (Figure 4a).

To further characterize the mechanism of transcriptional regulation of ferritin, cells were pre-treated with the protein synthesis inhibitor cycloheximide prior to treatment with iron or SNP. In these samples, a dramatic decrease of mRNA levels was observed, even though mRNA levels were still higher than control levels. It is thus concluded that *de novo* synthesis of one or more proteins is required for the accumulation ferritin transcript induced by either iron or SNP (Figure 4b).

Discussion

Depending on whether it is in a free or a complexed form, iron can act as either a pro- or antioxidant, respectively (Bowler *et al.*, 1992). The concentration of iron in the free form is finely regulated by modulation of ferritin levels (Briat and Lobreaux, 1997; Briat *et al.*, 1999; Harrison and Arosio, 1996; Theil, 1987). Indeed, iron is a strong inducer of ferritin in all plant systems tested (Fobis-Loisy *et al.*, 1995; Gaymard *et al.*, 1996; Lobreaux *et al.*, 1993; Lobreaux *et al.*, 1995).

The perturbation of ferritin levels in plants is not without effects. For example, overexpression of soybean ferritin in *Nicotiana tabacum* reduces the concentration of tissue chlorophyll by 20%. This causes ultrastructural disorganization in chloroplasts (Van Wuytswinkel *et al.*, 1999) and also makes plants more sensitive to photoinhibition of photosystem I (Murgia *et al.*, 2001). Most likely, illegitimate sequestration of iron takes place, thus hindering processes that require free, readily available iron, like chlorophyll

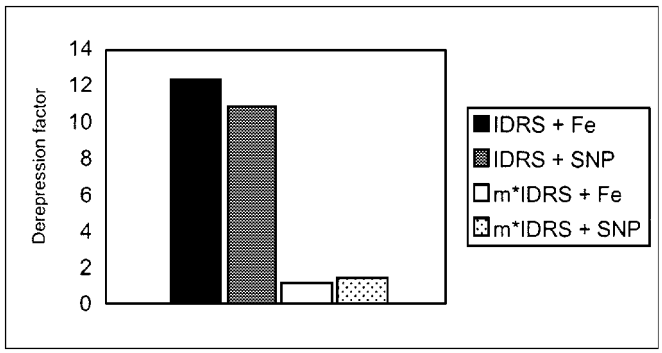

Figure 3. NO induces ferritin transcript accumulation via the IDRS sequence.
Leaves from 20-day-old-plants (At1400m* IDRS and At1400IDRS) were infiltrated with 1 mM SNP, 500 μM Fe-citrate or water (as control) and kept for 7 h in the dark. Protein extracts were prepared from four different leaves/samples collected from four different plants, and GUS activities were measured. The derepression factor corresponds to the ratio between GUS activities from SNP- or iron-treated plants to control plants. Data correspond to the mean values obtained from two independent experiments.

synthesis, chloroplast development and photosystem repair after photoinhibitory stress.

Understanding how transcription of the plant ferritin gene is regulated and what signaling molecules act downstream of iron in the signaling cascade has been the subject of research for several years. Indirect evidences suggested that NO is likely to be a down-regulator of ferritin synthesis. This possibility is based on studies using the NO donors NOC-9 and SIN-1, which inhibit tobacco aconitase (Navarre *et al.*, 2000). When inhibited by animal aconitase, NO is converted to IRP1, which prevents its translation upon binding to ferritin IRE sequences (Gardner *et al.*, 1997). Therefore, down regulation of ferritin mediated by NO was also anticipated in plants. However, plant ferritins are mainly regulated at the transcriptional level and although plant aconitases show high homology with the mammalian aconitases, especially in the IRP region, no IREs are present in plant ferritin genes.

We have found that the NO-donor SNP, which releases NO^+ *in vivo* (Bogdan, 2001; Kim and Ponka, 2000), causes a massive increase in ferritin transcript levels and a moderate increase in protein levels. NO induces ferritin transcription not simply by mimicking an 'iron-loading' effect, that is by releasing iron from complexes or from iron-proteins. If this were the case, a NO scavenger would not prevent exogenous iron from inducing ferritin accumulation, nor would iron-depleted cells be still able to accumulate ferritin when stimulated by NO. Our data indicate that NO is able to bypass iron and put NO downstream of iron itself, the most significant ferritin inducer, in the signaling cascade leading to accumulation of ferritin, via the IDRS sequence. This is supported also by the findings that nitrate reductase can produce NO (Yamasaki and Sakihama, 2000) and that ferritin accumulates in the nodules of nitrate-fed, dark-stressed bean and pea plants (Matamoros *et al.*, 1999; Ragland and Theil, 1993).

Additionally, in animals the IRP1/aconitase activity is not the main and sole regulator of ferritin levels. In the past it has been assumed that NO should always cause a reduction of ferritin levels in vertebrate cells by up-regulating IRP1 activity, thus reducing translation of ferritin and causing a likely enlargement of the LIP. However, recent findings show that NO can cause a specific down-regulation of IRP2 and induce ferritin translation (Bogdan, 2001; Bouton *et al.*, 1998; Cairo and

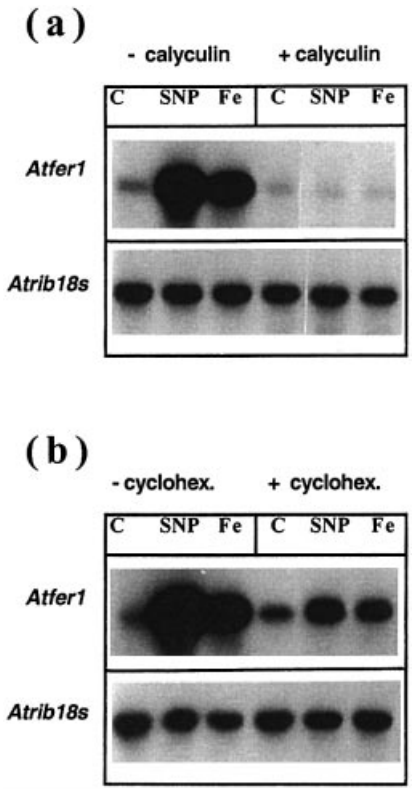

Figure 4. NO requires active protein synthesis and ser/thr phosphatase activity to induce ferritin transcript accumulation.
(a) Northern blot analysis of *Arabidopsis* suspension cultured cells, 3 h after treatment with 5 mM SNP, 100 μM Fe-citrate, in the presence or absence of 500 nM calyculin A. C represents the control (untreated cells). Five μg total RNA was loaded in each lane. Filters were hybridized with either the ferritin *Atfer1* probe (upper panel) or the constitutively expressed *Atrib18s* probe (lower panel).
(b) Northern blot analysis of *Arabidopsis* suspension cultures, 3 h after treatment with 5 mM SNP or 100 μM Fe-citrate, in the presence or absence of 20 μg ml^{-1} cycloheximide.

Pietrangelo, 2000; Recalcati *et al.*, 1998). The oxidized form of NO, namely NO^+, is responsible for the down-regulation of IRP2 (Kim and Ponka, 1999) and its ultimate degradation (Kim and Ponka, 2000). It has been proposed that, whereas the radical $NO^·$ activates IRP1 by disruption of the [4Fe-4S] cluster (Gardner *et al.*, 1997; Wardrop *et al.*, 2000), NO^+ down-regulates IRP2 by S-nitrosylation of sulfhydryl groups involved in binding to the IRE (Bogdan, 2001; Kim and Ponka, 2000). Protein S-nitrosylation by reactive nitrogen species has already been demonstrated (Stamler *et al.*, 1992; Stamler, 1994).

Although formal demonstration of the proposed mechanisms is lacking (Bogdan, 2001), the fact that NO can cause either inhibition or induction of ferritin synthesis in vertebrate cells can in part be explained by considering which form of NO prevails, NO^+ or $NO^·$. Independently of the prevailing active form of NO, under our experimental conditions it is clear that NO plays a role in the regulation of iron homeostasis in plants. The next challenge will be to identify the factor, most likely proteic (Petit *et al.*, 2001), which directly interacts with the IDRS sequence and is regulated by NO.

Experimental procedures

Plant growth

The Arabidopsis Col-0 line, kindly provided by the Arabidopsis Biological Resource Center was seeded in sterilized soil (Technic n.1, Dueemme, NL, USA) on Aratrays (BetaTech, Ghent, Belgium); seeds were vernalized at 4°C for 3–4 days to provide optimal germination. Plants were watered with deionized water and were grown at 100 μmol photons m^{-2} sec^{-1} at 21–25°C with a 14 h light/10 h dark photoperiod.

Infiltration of Arabidopsis leaves

Leaves of thoroughly watered 21–30-day-old plants were infiltrated using a 1-ml syringe, without a needle, at the abaxial leaf page. Infiltrated plants were kept in the dark and leaves were cut and collected at different time points (for extraction of RNA or protein, three leaves from three different rosettes were used for each time-point; each rosette was cut only once). Leaves were frozen in liquid N_2 for RNA or protein extraction.

Preparation of stock solutions

Stock solutions were prepared in the following manner: 100 mM SNP in H_2O; 50 mM Fe-citrate by mixing equal volumes of 100 mM $FeSO_4$ (in 0.06 N HCl) and 200 mM Na-citrate; 100 mM CPTIO in H_2O; 2 mg ml^{-1} cycloheximide in H_2O; 50 μM calyculin A in 100% ethanol. H_2O_2 was prepared as a 1M stock solution in H_2O, titrated in excess with 1 N H_2SO_4 containing 0.1 N $KMnO_4$; all stock solutions were stored at –20°C.

Treatments of Arabidopsis suspension cultured cells

Arabidopsis suspension cultured cells (Landsberg) were grown as previously described (Curti *et al.*, 1993). Seven-day-old subcultures, from 1 ml cells inoculum in 90 ml growth medium, were used for the experiments. Cell suspensions were filtrated through a 25-μm nylon cloth with suction; cells were then rapidly weighed and aliquoted at 100 mg per well (Transwell, 24 mm diameter, 8 μm filter pore size, Corning Costar Corp., Corning, NY, USA) containing 3 ml wash buffer. Experiments were carried out at room temperature (25°C), under gentle shaking (80 r.p.m.). Cells were washed three times (10, 15, 15 min long) in wash buffer (3 ml per well). After the last wash, the buffer was replaced with experiment buffer (3 ml per well) and the cells were allowed to recover from stress 30 min before any addition. CPTIO was added to cells 5 min before further treatments. Calyculin A and cycloheximide were added 90 min before further treatments. DFO (500 μM) or ferrozine (1 mM) were added to the cells 20 h before further treatments. Cells were collected after 3 h and frozen in liquid nitrogen for RNA extraction. Wash buffer: 0.2 mM $CaSO_4$, 0.5 mM MES (2-(N-morpholino) ethanesulfonic acid), at pH 6.0 with bis-tris-propane (BTP). Experiment buffer: 0.2 mM $CaSO_4$, 1 mM K_2SO_4, 10 mM MES, at pH 6.0 with BTP.

The iron chelants DFO (500 μM) or ferrozine (1 mM) were added directly to the flasks containing the suspension cultured cells 20 h before further experiments.

Northern blots

RNA was extracted using Trizol for both leaves and cells (Trizol reagent, Gibco BRL Life Technologies, http://www.lifetech.com). 5 μg RNA per sample was used in RNA gel electrophoresis (Murgia *et al.*, 1995). RNA was then blotted overnight onto a nylon membrane Hybond-N^+ (Amersham, Biosciences, Little Chalfont, Buckinghamshire, UK), crosslinked to the membrane with 2 h baking at 80°C and hybridized with a radioactive probe using the Rediprimell Amersham kit. The *Arabidopsis* ferritin probe was a Not1-EcoR1 fragment (about 600 bp) from the *Atfer1* cDNA subcloned in pBluescript (Gaymard *et al.*, 1996).

Western blotting

Crude protein extracts were prepared by placing 0.5 g ground leaf tissue in 1.0 ml of extraction buffer (50 mM Tris/HCl pH 8.0, 10% sucrose, 5% SDS, 1 mM EDTA; 5% β-mercaptoethanol with 1 mM phenylmethylsulfonylfluoride and 0.01% bathophenantroline added just before use). Samples were then mixed and centrifuged at room temperature for 30 min at 20 000 *g*. Protein concentration was measured using the Lowry procedure (Sigma Diagnostic Protein Assay Kit), following the manufacturer's instructions. The primary antibody was a rabbit polyclonal against pea ferritin (diluted 1 : 500), while the secondary antibody was goat anti-rabbit conjugated with horseradish peroxidase (diluted 1 : 20 000). Signals were detected with the Pierce Supersignal cheminuminescent substrate.

GUS assay

Protein extracts were prepared by homogenizing four leaves per sample with a mortar and pestle in the presence of 50–100 mg sand in 500 μl EB buffer (50 mM sodium phosphate buffer, pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2-EDTA, 0.1% sodium N-lauroylsarcosine, and 0.1% Triton X-100). The homogenate was centrifuged for 1 min at 8000 r.p.m and 400 μl of supernatant extract was transferred to a clean tube and used for GUS assay. Reactions for the GUS assay were performed in a final volume of

1 ml and contained 500 μl of 2 mM MUG (4-methylumbelliferyl β-D-glucuronide), 480 μl EB, and 20 μl protein extract. As soon as the extract was added, the mixture was quickly vortexed and 100 μl aliquots were withdrawn at regular time intervals (1, 3, 5 min) and added to fresh tubes containing 1.9 ml stop solution (0.2 M Na_2CO_3). Fluorescence was measured in a fluorometer (Hoefer Scientific Instruments, San Francisco, CA, USA) calibrated at 50 nM MU (4-methylumbelliferone) = 500 fluorescence units. Protein concentration in the extracts was measured using the Lowry procedure (Sigma Diagnostic Protein Assay Kit), following the manufacturer's instructions. GUS activity was calculated as nmol MU min^{-1} mg^{-1} protein.

Acknowledgements

This work was supported by MURST 2000 in the framework of the program 'Nitric oxide and plant resistance to pathogens'.

We thank Prof Albergoni's group for providing *Arabidopsis* suspension cultured cells, Dr Jean François Briat for donating polyclonal antibodies against pea ferritin and Dr Stephan Lobreaux for donating the At1400m*IDRS and At1400IDRS *Arabidopsis* transformed lines.

References

Andrews, S.C., Smith, J.M.A., Yewdall, J.R., Guest, J.R. and Harrison, P.M. (1991) Bacterioferritins and ferritins are distantly related in evolution. *FEBS Lett.* **293**, 164–168.

Barford, D. (1996) Molecular mechanisms of the protein serine/threonine phosphatases. *Trends Biochem. Sci.* **21**, 407–412.

Beligni, M.W. and Lamattina, L. (2000) Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. *Planta*, **210**, 215–221.

Bogdan, C. (2001) Nitric oxide and the regulation of gene expression. *Trends Cell Biol.* **11**, 66–75.

Bouton, C., Oliveira, L. and Drapier, J.C. (1998) Converse modulation of IRP1 and IRP2 by immunological stimuli in murine RAW 264.7 macrophages. *J.Biol. Chem.* **273**, 9403–9408.

Bowler, C., van Montagu, M. and Inzé, D. (1992) Superoxide dismutase and stress tolerance. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 83–116.

Briat, J.F. and Lobreaux, S. (1997) Iron transport and storage in plants. *Trends Plant Sci.* **2**, 187–192.

Briat, J.F., Lobreaux, S., Grignon, N. and Vansuyt, G. (1999) Regulation of plant ferritin synthesis: how and why. *Cell. Mol. Life. Sci.* **56**, 155–166.

Cairo, G. and Pietrangelo, A. (2000) Iron regulatory proteins in pathobiology. *Biochem. J.* **352**, 241–250.

Carter, P.J., Nimmo, H.G., Fewson, C.A. and Wilkins, M.B. (1990) *Bryophyllum fedtschenkol* protein phosphatase type 2A can dephosphorylate phosphoenolpyruvate carboxylase. *FEBS Lett.* **263**, 233–236.

Curti, G., Massardi, F. and Lado, P. (1993) Synergistic activation of plasma membrane H^+-ATPase in *Arabidopsis thaliana* by turgor decrease and by fusicoccin. *Physiol. Plant.* **87**, 592–600.

Delledonne, M., Murgia, I., Ederle, D., Sbicego, P.F., Biondani, A., Polverari, A. and Lamb, C. (2002a) Reactive Oxygen Intermediates modulate nitric oxide signaling in the plant hypersensitive disease resistance response. *Plant Physiol. Biochem.* (in press.)

Delledonne, M., Polverari, A. and Murgia, I. (2002b) Nitric oxide-mediated signaling functions and changes in gene expression during the plant hypersensitive response. *Antiox. Redox Signal.* (in press.)

Delledonne, M., Xia, Y., Dixon, R.A. and Lamb, C. (1998) Nitric oxide functions as signal in plant desease resistance. *Nature*, **394**, 585–588.

Delledonne, M., Zeier, J., Marocco, A. and Lamb, C. (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proc. Natl Acad. Sci. USA*, **98**, 13454–13459.

Durner, J., Gow, A.J., Stamler, J. and Glazebook, J. (1999) Ancient origins of nitric oxide signaling in biological systems. *Proc. Natl Acad. Sci. USA*, **96**, 14206–14207.

Durner, J., Wendehenne, D. and Klessig, D.F. (1998) Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc. Natl Acad. Sci. USA*, **95**, 10328–10333.

Fobis-Loisy, I., Aussel, L. and Briat, J.F. (1996) Post-transcriptional regulation of plant ferritin accumulation in response to iron as observed in the maize mutant. *Ys1. FEBS Lett.* **397**, 149–154.

Fobis-Loisy, I., Loridon, K., Lebrun, M. and Briat, J.F. (1995) Stucture and differential expression of two maize ferritin genes in response to iron and abscissic acid. *Eur. J. Biochem.* **231**, 609–619.

Garbers, C., Delong, A., Deruere, J., Bernasconi, P. and Soll, D. (1996) A mutation in protein phosphatase 2A regulatory subunit A affects auxin transport in *Arabidopsis thaliana*. *EMBO J.* **15**, 2115–2124.

Gardner, P.R., Costantino, G., Szabò, C. and Salzman, A.L. (1997) Nitric oxide sensitivity of the aconitases. *J.Biol. Chem.* **272**, 25071–25076.

Gaymard, F., Boucherez, J. and Briat, J.F. (1996) Characterization of a ferritin mRNA from *Arabidopsis thaliana* accumulated in response to iron through an oxidative pathway independent of abscissic acid. *Biochem. J.* **318**, 67–73.

Guerinot, M.L. and Yi, Y. (1994) Iron: nutritious, noxious and not ready available. *Plant Physiol.* **104**, 815–820.

Harrison, P.M. and Arosio, P. (1996) The ferritins: molecular properties, iron storage function and cellular regulation. *Biochem. Biophys. Acta*, **1275**, 161–203.

Hausladen, A. and Stamler, J. (1998) Nitric oxide in plant immunity. *Proc. Natl Acad. Sci. USA*, **95**, 10345–10347.

Hentze, M.W. and Kühn, L.C. (1996) Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide and oxidative stress. *Proc. Natl Acad. Sci. USA*, **93**, 8175–8182.

Kim, S. and Ponka, P. (1999) Control of transferrin receptor expression via nitric-oxide mediated modulation of iron regulatory protein-2. *J.Biol. Chem.* **274**, 33035–33042.

Kim, S. and Ponka, P. (2000) Effects of interferon-γ and lipopolysaccharide on macrophage iron metabolism are mediated by nitric oxide induced degradation of iron-regulatory protein-2. *J.Biol. Chem.* **275**, 6220–6226.

Klessig, D.F., Durner, J., Noad, R., Navarre, D.A., Wendehenne, D., Kumar, D., Zhou, J.M., Shah, J., Zhang, S., Kachroo, P., Trifa, Y., Pontier, D., Lam, E. and Silva, H. (2000) Nitric Oxide and salicylic acid signaling in plant defence. *Proc. Natl Acad. Sci. USA*, **97**, 8849–8855.

Laulhere, J.P. and Briat, J.F. (1993) Iron release and uptake by plant ferritin: effects of pH, reduction and chelation. *Biochem. J.* **290**, 693–699.

Leshem, Y.Y. and Pinchasov, Y. (2000) Non-invasive photoacoustic spectroscopic determination of relative endogenous nitric oxide and ethylene content stoichiometry during the ripening of strawberries *Fragaria anannasa* and avocados *Persea amaracana*. *J.Exp. Bot.* **51**, 1471–1473.

Leshem, Y.Y., Wills, R.B.H. and Ku, V.V.V. (1998) Evidence for the function of the free radical gas nitric oxide ($NO^{\cdot}$) as an endogenous maturation and senescence regulating factor in higher plants. *Plant Physiol. Biochem.* **36** (11), 825–833.

Lobreaux, S., Hardy, T. and Briat, J.F. (1993) Abscissic acid is involved in the iron-induced synthesis of maize ferritin. *EMBO J.* **12**, 651–657.

Lobreaux, S., Massenet, O. and Briat, J.F. (1992a) Iron induces ferritin synthesis in maize plantlets. *Plant Mol. Biol.* **19**, 563–575.

Lobreaux, S., Thoiron, S. and Briat, J.F. (1995) Induction of ferritin synthesis in maize leaves by an iron-mediated oxidative stress. *Plant J.* **8**, 443–449.

Lobreaux, S., Yewdall, S., Briat, J.F. and Harrison, P.M. (1992b) Amino-acid sequence and predicted three-dimentional structure of pea seed (*Pisum sativum*) ferritin. *Biochem. J.* **288**, 931–939.

Mata, C.G. and Lamattina, L. (2001) Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. *Plant Physiol.* **126**, 1196–1204.

Matamoros, M.A., Baird, L.M., Escuredo, P.R., Dalton, D.A., Minchin, F.R., Iturbe-Ormaetxe, I., Rubio, M.C., Moran, J.F., Gordon, A.J. and Becana, M. (1999) Stress-induced legume root nodule senescence. Physiological, biochemical and structural alterations. *Plant Physiol.* **121**, 97–111.

Murgia, I., Briat, J.F., Tarantino, D. and Soave, C. (2001) Plant ferritin accumulates in response to photoinhibition but its ectopic overexpression does not protect against photoinhibition. *Plant Physiol. Biochem.* **39**, 1–10.

Murgia, I., Maciver, S. and Morandini, P. (1995) An actin-related protein from *Dictyostelium discoideum* is developmentally regulated and associated with mitochondria. *FEBS Lett.* **360**, 235–241.

Nathan, C. (1995) Natural resistance and Nitric oxide. *Cell*, **82**, 873–876.

Navarre, D.A., Wendehenne, D., Durner, J., Noad, R. and Klessig, D. (2000) Nitric oxide modulates the activity of tobacco aconitase. *Plant Physiol.* **122**, 573–582.

Noctor, G. and Foyer, C. (1998) Ascorbate and glutathione: keeping active oxygen under control. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 249–279.

Petit, J.M., van Wuytswinkel, O., Briat, J.F. and Lobreaux, S. (2001) Characterization of an iron-dependent regulatory sequence involved in the transcriptional control of *Atfer1* and *Zmfer1* plant ferritin genes by iron. *J.Biol. Chem.* **276** (8), 5584–5590.

Peyret, P., Perez, P. and Alric, M. (1995) Structure, genomic organization and expression of the *Arabidopsis thaliana* aconitase gene. *J.Biol. Chem.* **270**, 8131–8137.

Prouhdon, D., Wei, J. and Theil, E.C. (1996) Ferritin gene organization: differences between plants and animals suggest possible kingdom-specific selective constraints. *J.Mol. Evol.* **42**, 325–336.

Ragland, M. and Theil, E.C. (1993) Ferritin (mRNA, protein) and iron concentrations during soybean nodule development. *Plant Mol. Biol.* **21**, 555–560.

Recalcati, S., Taramelli, D., Conte, D. and Cairo, G. (1998) Nitric oxide mediated induction of ferritin synthesis in J774 macrophages by inflammatory cytokines; role of selective iron regulatory protein-2 downregulation. *Blood*, **91**, 1059–1066.

Savino, G., Briat, J.F. and Lobreaux, S. (1997) Inhibition of the iron-induced *ZmFer1* Maize ferritin gene expression by antioxidants and serine/threonine phosphatase inhibitors. *J.Biol. Chem.* **272**, 33319–33326.

Schmidt, H.H. and Walter, U. (1994) NO at work. *Cell*, **78**, 919–925.

Smith, R.D. and Walker, J.C. (1996) Plant protein phosphatases. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 101–125.

Stamler, J.S. (1994) Redox signaling: Nitrosylation and related target interactions of nitric oxide. *Cell*, **78**, 931–936.

Stamler, J.S., Singel, D.J. and Loscalzo, J. (1992) Biochemistry of nitric oxide and its redox-activated forms. *Science*, **258**, 1898–1902.

Stamler, J.S., Toone, E.J., Lipton, S.A. and Sucher, N.J. (1997) (S) NO signals: translocation, regulation, and a consensus motif. *Neuron*, **18**, 691–696.

Theil, E.C. (1987) Ferritin: structure, gene regulation, and cellular function in animals, plants and microorganisms. *Ann. Rev. Biochem.* **56**, 289–315.

Van Wuytswinkel, O., Vansuyt, G., Grignon, N., Fourcroy, P. and Briat, J.F. (1999) Iron homeostasis alteration in transgenic tobacco overexpressing ferritin. *Plant J.* **17**, 93–97.

Wardrop, S.L., Watts, R.N. and Richardson, D.R. (2000) Nitrogen monoxide activates Iron Regulatory Protein 1 RNA-binding activity by two possible mechanisms: effect of the [4Se-4S] cluster and iron mobilization from cells. *Biochem.* **39**, 2748–2758.

Wei, J. and Theil, E. (2000) Identification and characterization of a plant regulatory element in the ferritin gene of a plant (soybean). *J.Biol. Chem.* **275** (23), 17488–17943.

Wendehenne, D., Pugin, A., Klessig, D., F. and Durner, J. (2001) Nitric oxide: comparative synthesis and signalling in animal and plant cells. *Trends Plant Sci.* **6** (4), 177–183.

Yamasaki, H. and Sakihama, Y. (2000) Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. *FEBS Lett.* **468**, 89–92.