An iron-induced nitric oxide burst precedes ubiquitin-dependent protein degradation for Arabidopsis Atfer1 ferritin gene expression.

Nicolas Arnaud, Iréne Murgia, Jossia Boucerez, Jean-François Briat, Françoise Cellier, Frédéric Gaymard

To cite this version:
Nicolas Arnaud, Iréne Murgia, Jossia Boucerez, Jean-François Briat, Françoise Cellier, et al.. An iron-induced nitric oxide burst precedes ubiquitin-dependent protein degradation for Arabidopsis Atfer1 ferritin gene expression. Journal of Biological Chemistry, American Society for Biochemistry and Molecular Biology, 2006, 281 (33), pp.23579-23588. 10.1074/jbc.M602135200. hal-00091275

HAL Id: hal-00091275
https://hal.archives-ouvertes.fr/hal-00091275
Submitted on 30 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
An Iron-induced Nitric Oxide Burst Precedes Ubiquitin-dependent Protein Degradation for Arabidopsis AtFer1 Ferritin Gene Expression*

Received for publication, March 7, 2006, and in revised form, May 2, 2006 Published, JBC Papers in Press, June 16, 2006, DOI 10.1074/jbc.M602135200

Nicolas Arnaud†1, Irene Murgia§, Jossia Boucherez†, Jean-François Briat†, Françoise Cellier†, and Frédéric Gaymard‡2

From the †Laboratoire de Biochimie et Physiologie Moléculaire des Plantes, UMR 5004 Agro-M/CNRS/INRA/UMII, Bat 7, 2 place Viala, 34060 Montpellier Cedex 1, France and the §Sezione di Fisiologia e Biochimica delle Piante, Dipartimento di Biologia, Università degli Studi di Milano, via Celoria 26, 20133 Milano, Italy

Ferritins play an essential role in iron homeostasis by sequestering iron in a bioavailable and non-toxic form. In plants, ferritin mRNAs are highly and quickly accumulated in response to iron overload. Such accumulation leads to a subsequent ferritin protein synthesis and iron storage, thus avoiding oxidative stress to take place. By combining pharmacological and imaging approaches in an Arabidopsis cell culture system, we have identified several elements in the signal transduction pathway leading to the increase of AtFer1 transcript level after iron treatment. Nitric oxide quickly accumulates in the plastids after iron treatment. This compound acts downstream of iron and upstream of a PP2A-type phosphatase to promote an increase of AtFer1 mRNA level. The AtFer1 gene transcription has been previously shown to be repressed under low iron conditions with the involvement of the cis-acting element iron-dependent regulatory sequence identified within the AtFer1 promoter sequence. We show here that the repressor is unlikely a transcription factor directly bound to the iron-dependent regulatory sequence; such a repressor is ubiquitinated upon iron treatment and subsequently degraded through a 26 S proteasome-dependent pathway.

As the major cofactor of proteins involved in essential processes like photosynthesis, respiration, DNA replication, or nitrogen fixation, iron is an essential element for life. Nonetheless, in the free ionic form, iron is toxic as it can catalyze the formation of reactive oxygen species through the Fenton reaction. These reactive oxygen species damage the cell membranes, DNA, and proteins (1, 2). Thus, iron homeostasis has to be tightly regulated, to avoid starvation that impairs the metabolism, and to avoid excess that may lead to cell death. Iron homeostasis is strongly dependent on ferritins, which are iron-storage proteins, found in bacteria, animals, and plants. Plant and animal ferritin structures are very similar, and are formed by 24 subunits arranged to form a hollow sphere able to sequester iron in a non-toxic and bioavailable form (3).

In animals, ferritin synthesis is mainly regulated at the post-transcriptional level (3, 4). Ferritin mRNAs contain iron-responsive elements in their 5′-untranslated regions that function as binding sites for two related trans-acting factors, namely iron regulatory proteins IRP1 and IRP2. When bound to the iron-responsive element in the ferritin mRNA, the IRP inhibit translation of the transcript (4). IRP1 is a bifunctional protein that when iron is abundant possesses a 4Fe-4S cluster and acts as cytoplasmic aconitase. When iron levels are low, the 4Fe-4S cluster disassembles and the apoprotein acquires IRP3 activity, thus repressing ferritin translation. High levels of iron lead to the 4Fe-4S cluster reconstitution and therefore the protein aconitase activity. In contrast to IRP1, IRP2 cannot assemble a iron-sulfur cluster and lacks aconitase activity. IRP2 shares about 60% amino acid sequence identity with IRP1, but differs only in having a 73-amino acid insertion in its N-terminal region. This region contains a cysteine-rich sequence responsible for targeting the protein for degradation via the ubiquitin-proteasome pathway when cellular iron level is high (5, 6). NO has been shown to play an important role in iron metabolism by modulating both IRP1 and IRP2 activities (7, 8). Exposure to NO was shown to disassemble the iron-sulfur cluster of IRP1, promoting binding to ferritin mRNA (4, 9). By contrast, IRP2 binding to iron-responsive elements is negatively regulated by NO (10–12). An oxidized form of NO, the nitrosonium ion NO+ (11, 13) may cause the S-nitrosylation of a cysteine found in the Fe2+-dependent degradation domain of IRP2, leading to a subsequent and specific down-regulation of IRP2 by the ubiquitin/26 S proteasome pathway (14, 15).

Plant ferritins can be found in mitochondria (16), but in contrast to animal cells, they have never been observed in the cyto-

* This work was supported by Institut National de la Recherche Agronomique and Centre National de la Recherche Scientifique, Action Concertée Incitative “Biologie Cellulaire Moléculaire et Structurale” Grant BCM166 from the Ministère de l’Education Nationale, de l’Enseignement Supérieur et de la Recherche. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a thesis fellowship from the Ministère de l’Education Nationale, de l’Enseignement Supérieur et de la Recherche.

‡ To whom correspondence should be addressed. Tel.: 33-499-61-29-32; Fax: 33-467-52-57-37; E-mail: gaymard@ensam.inra.fr.

§ Supported by a thesis fellowship from the Ministère de l’Education Nationale, de l’Enseignement Supérieur et de la Recherche.

The abbreviations used are: IRP, iron regulatory protein; cPTIO, 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazol-1-yl-oxy-3-oxide; DAF-FM DA, 4-amino-5-methylamino-2′,7′-dihydrofluorescein diacetate; IDR5, iron-dependent regulatory sequence; l-NMMA, Nω-monomethyl-l-arginine; PP2A, protein phosphatase type 2A; OA, okadaic acid; SNP, sodium nitroprusside; NO, nitric oxide; MES, 4-morpholineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; DTT, dithiothreitol; NR, nitrate reductase; NOS, nitric-oxide synthase; ABA, abscisic acid.
Regulation of Ferritin by NO and Protein Degradation

plasm, and their main location is in the plastids (17). In addition, their synthesis is regulated at the transcriptional level in response to iron excess (17, 18), and not at the translational level as described above for animal cells. In plants, ferritin mRNA abundance has been shown to be regulated by several environmental factors including iron (17, 19–21), H₂O₂ (22), photoinhibition (23), pathogen attacks (24), by the stress hormone ABA (25), and by NO donors or scavengers (26, 27). Experiments based on serial deletions and site-directed mutagenesis of maize ZmFer1 and Arabidopsis AtFer1 ferritin promoter sequences allowed to identify a 15-bp cis-acting element necessary for the iron-dependent regulation of the transcription of these genes (18). This sequence, named IDRS, for cis element necessary for the iron-dependent regulation of the transcription of these genes (18). This sequence, named IDRS, for iron-dependent regulatory sequence, has been shown to be involved in the repression of ZmFer1 and AtFer1 gene expression under iron-deficient conditions (18, 28). Thus, iron addition leads to the de-repression of ZmFer1 and AtFer1 gene expression rather than to their induction.

Despite the growing number of physiological conditions reported to date leading to plant ferritin synthesis, little is known about the regulatory molecules acting downstream of iron. By using an Arabidopsis cell culture system, we show in this work that iron excess and oxidative stress, mimicked by exogenous H₂O₂ application, promote AtFer1 gene expression through two independent and additive pathways. We show also that iron application leads to a rapid NO burst in the plastids of the cell. This NO accumulation, which does not involve NOS1 nor nitrate reductase activities, is leading to AtFer1 de-repression. The factor that represses AtFer1 transcription under iron-deficient conditions is ubiquitinated and degraded by a 26 S proteasome-dependent pathway after iron application. This repressor is not a transcription factor directly bound to the IDRS present in the AtFer1 promoter region.

EXPERIMENTAL PROCEDURES

Plant Cell Culture—Arabidopsis thaliana L. (Columbia ecotype) suspension cells were grown at 24 °C under continuous light (100 μmol m⁻² s⁻¹) on a rotating table (60 rev/min) in a medium containing 20 mM KNO₃, 1.2 mM CaCl₂, 450 μM MgSO₄, 375 μM KH₂PO₄, 60 μM Na₂HPO₄, 40 μM NaH₂PO₄, 40 μM MnSO₄, 30 μM H₂BO₃, 25 μM glycine, 10 μM ZnSO₄, 5 μM Fe(III)-EDTA, 4 μM nicotinic acid, 2.5 μM pyridoxine-HCl, 1.5 μM KI, 1.2 μM thiamine-HCl, 300 μM Na₂MoO₄, 100 μM ANA, 30 μM CoCl₂, 30 μM CuSO₄, 0.1 g liter⁻¹ casein hydrolysate, 0.1 g liter⁻¹ myo-inositol, 15 g liter⁻¹ sucrose, pH 5.7. Cells were subcultured with a 1/10 dilution factor every 7 days. Experiments were carried out 1 week after subculture.

Seeds from A. thaliana L. (Columbia ecotype), atnos1 (29), and g'4-3 (30) mutants were surface-sterilized by immersion in a 4% (w/v) Bayrochlor, 50% ethanol solution for 20 min. Seeds were washed three times with ethanol and left to dry in sterile conditions. Seedlings were grown in 100 ml of half-strength Murashige and Skoog medium (Sigma), pH 5.7, supplemented with 1% sucrose, 0.5 g liter⁻¹ MES, and 50 μM Fe(III)-EDTA. After 1 week of culture at 24 °C under continuous light (100 μE m⁻² s⁻¹) and shaking (60 rpm), medium was discarded and replaced by 100 ml of fresh medium. Plants were grown 4 additional days in these conditions before treatments.

Chemicals—One volume of a 100 mM FeSO₄ stock solution in 0.06 M HCl was mixed with 1 volume of 200 mM Na₃-citrate for a concentration of 50 mM FeSO₄, 100 mM Na₃-citrate. This mixture was used at final concentration of 300 μM FeSO₄, 600 μM Na₃-citrate in the culture medium. Except where indicated, all chemicals were purchased from Sigma. Okadaic acid and cycloheximide were dissolved in ethanol and used at final concentrations of 250 nM and 100 μM, respectively. MG132 was dissolved in Me₂SO and used at a final concentration of 50 μM. Pefabloc (Roche Applied Science), ePTIO, 1- NMMA, and SNP were dissolved in sterile water and used at final concentrations of 100 μM, and 1, 5, and 2.5 mM, respectively. After treatments, cells were filtered or plantlets were collected and immediately frozen in liquid nitrogen and stored at −80 °C.

Microscopy—NO imaging was performed by using 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA, Molecular Probes) dissolved in Me₂SO at a stock concentration of 5 mM. For confocal laser-scanning microscopy, cells were loaded with 5 μM DAF-FM DA for 20 min. Then a solution containing 300 μM FeSO₄, 600 μM Na₃-citrate or 300 μM K₃SO₄, 600 μM Na₂-citrate was added. The cell suspension (30 μl) was transferred on the slide. After overlaying by the glass slide, the slides were placed under the microscope, and the images were taken within 5 min on the same cells for each treatment. Settings and laser of the Zeiss Axiovert 100M inverted microscope were as described previously (31). Microscope, laser, and photomultiplier settings were held constant during the course of an experiment to obtain comparable images. Images were processed and analyzed using the Zeiss LSM 510 software.

RNA Preparation and Analysis—Total RNA were extracted from cells and plantlets as indicated in Ref. 25. For Northern blot analysis, 10 μg of total RNA were loaded in each lane, separated by electrophoresis through a 1.2% (w/v) agarose/formaldehyde gel, and blotted onto a nylon membrane (Hybond N; Amersham Biosciences). Hybridizations with 32P-labeled probes were performed overnight at 42 °C in the presence of 50% formamide (32). After washes, filters were exposed for a few hours at −80 °C to Fuji Medical X-Ray film Super RX (Fujifilm) with an intensifying screen. AtFer1 mRNA relative abundance was determined by measuring hybridization signal intensities of AtFer1 and EF1α on the same blot. Quantifications were performed with the Imager Reader Bas-5000 software (Fuji). The AtFer1 mRNA relative abundance was defined as the ratio of AtFer1 and EF1α signal intensities.

Protein Preparation and Analysis—Total protein extracts were prepared from 1 g of each sample as described (33). Protein concentration was determined according to Schaffner and Weissmann (34) using bovine serum albumin as standard. Proteins were subjected to electrophoresis on a 13% polyacrylamide, 0.1% SDS gel according to Laemmli (35). After electrophoresis, the gel was fixed and stained with Coomassie Brilliant Blue, then gels were scanned and analyzed using the ImageMaster 2D Platinum 5.0 software.

Preparation of Nuclear Extracts—All procedures were carried out at 4 °C. Frozen cells (50 g) were ground in a Waring
Blender in 300 ml of homogenization buffer (250 mM sucrose, 10 mM NaCl, 25 mM Pipes, 5 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 20 mM β-mercaptoethanol, 0.1% Nonidet P-40, and 0.2 mM phenylmethylsulfonyl fluoride, pH 7.0). The homogenates were filtered through two layers of Miracloth (Calbiochem). Nuclei were recovered by centrifugation at 4,200 × g for 20 min at 4 °C, then were gently resuspended, and washed four times with homogenization buffer with subsequent centrifugations at 2,000 × g for 10 min, then at 1,500 × g for 10, 8, and 6 min. Nuclei were resuspended in a minimum volume of freezing buffer (100 mM NaCl, 50 mM Hepes, 10 mM KCl, 5 mM MgCl₂, 1 mM DTT, 0.5 μg ml⁻¹ leupeptin, and 50 μg ml⁻¹ antipain, 50% glycerol, pH 7.6), frozen in liquid nitrogen, and stored at −80 ºC until use. Nuclear extracts were prepared by thawing nuclei on ice and lysing by adjusting the NaCl concentration to 0.47 M with lysing buffer (2.5 M NaCl, 50 mM Hepes, 10 mM KCl, 5 mM MgCl₂, 1 mM DTT, 0.5 μg ml⁻¹ leupeptin, 50 μg ml⁻¹ antipain, 20% glycerol, pH 7.6), and then shaking at 4 ºC for 30 min. Chromatin was pelleted by centrifugation at 13,000 × g for 15 min, and the supernatant containing nuclear proteins was dialyzed for 4 h against dialysis buffer (20 mM Hepes, 0.2 mM EDTA, 1 mM DTT, 20% glycerol, pH 7.6). Nuclear proteins were concentrated with centrifugal filter devices (Amicon, Ultracel 10k). Protein concentration was determined according to Schaffner and Weissmann (34) using bovine serum albumin as standard. Nuclear extracts were frozen in liquid nitrogen, and stored at −80 ºC.

**DNA Probes and Labeling Reactions**—The specific probe used for AtFer1 detection consists in a chimeric fragment containing the 5’- and 3’-untranslated regions of the AtFer1 cDNA. The 5’-untranslated region was amplified with thermostable Pfu DNA polymerase (Promega) using primers 5’-GGTACCATATAAAACCTTCCTCCCTC-3’ and 5’-GAATTCCATCGCGATGTTGTTGTTGTC-3’ introducing KpnI and EcoRI sites at the 5’ and 3’ ends of the amplified fragment, respectively. The 3’-untranslated region was amplified using primers 5’-GAACAGAATTCGGACCTCTTATA-3’ and 5’-TAGAAACACTGTA-AAAACAAAACTCATTG-3’ introducing EcoRI and Spel sites at the 5’ and 3’ ends of the amplified fragment, respectively. The fragments were cloned at the corresponding sites in pBluescript and sequenced. The probe was obtained after digestion of the corresponding plasmids by BamHI and HindIII. The fragments were cloned in pBluescript at the corresponding sites and sequenced. For probes A, B, C, and D (see the location of the amplified fragments on Fig. 6), the primer located at the 5’ end of the amplified fragment is 5’-GGATCCAGCGAGTAGGA-AATA-3’. At the 3’ ends, the primers were 5’-CTCGAGAAAGGGCTGTGTGTCACGGTGG-3’, 5’-CTCGAGCCGTTGGATTGAGATCC-3’, 5’-CTCGAGTGGATGATAGGAGCG-3’, and 5’-CTCGAGATGTGTTGAACTGTGAG-3’ for probes A, B, and D, respectively. The probe E was obtained with primers 5’-GGATCCAGATTACGCTTAACTT-3’ and 5’-CTCGAGATCATCCTCTCCAAAATAGTGTGTGTC-3’. For labeling and competitions, the fragments were obtained by digestion of the corresponding plasmids by BamHI and Xhol and subsequent purification on agarose gel. For labeling, 100 ng of DNA was introduced in a medium containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 50 μg ml⁻¹ bovine serum albumin, dATP, dGTP, dTTP (600 μM each), 50 μCi of [³²P]dCTP and 10 units of Klenow fragment. After 30 min at room temperature, DNA was purified by phenol/chloroform extraction and ethanol precipitation. After a 15-min centrifugation at 10,000 × g at 4 ºC, the pellet was washed twice with 70% ethanol, dried, and re-suspended in 50 μl of water. Specific activity of the probe was determined by scintillation counting, and the probe was diluted to 20,000 cpm/μl.

**Mobility Shift Assay**—The mobility shift reaction was done in a volume of 30 μl using 1 μl of [³²P]labeled DNA fragment, 2 μg of poly(dI-dC), and 5 μg of nuclear protein in the fixation buffer (25 mM Hepes-KOH, 70 mM KCl, 1 mM EDTA, 1 mM DTT, 50% glycerol, pH 7.6). The binding reaction was performed for 30 min at room temperature prior to loading reactions onto 6% polyacrylamide non-denaturing gel in 45 mM Tris, 45 mM boric acid, 0.5 mM EDTA, 5% glycerol, pH 8.0. The gel was run at 120 V in 45 mM Tris, 45 mM boric acid, 0.5 mM EDTA, pH 8.0, buffer for ~6 h. After migration, the gel was dried during 2 h under vacuum at 80 ºC and exposed for a few hours at ~80 ºC to Fuji Medical X-Ray film Super RX (Fujifilm). For the binding competition assays, a 50-fold molar excess of unlabeled fragments was included in the reaction.

**RESULTS**

Iron and H₂O₂ Increase AtFer1 mRNA Abundance by Two Independent and Additive Pathways—Both maize ZmFer1 and Arabidopsis AtFer1 ferritin mRNA accumulates in response to iron treatment. This response, dependent of the cis-acting element IDRS (18), is antagonized by antioxidants like N-acetylcysteine and GSH (20, 22), indicating that an oxidative step is involved in the pathway leading to the iron-dependent ferritin mRNA increase in abundance. Furthermore, it has been previously reported that H₂O₂ treatment increases maize ZmFer1 and Arabidopsis AtFer1 mRNA abundance (21, 22). It can therefore be hypothesized that H₂O₂ could act in the iron/IDRS-dependent pathway, and this is the first point we analyzed. We checked whether H₂O₂ treatment could mimic the potential oxidative effect of iron overload. Arabidopsis cell cultures were treated either with 300 μM iron-citrate, 5 mM H₂O₂, or with both inducers. Above 300 μM iron-citrate or 5 mM H₂O₂, no significant difference in AtFer1 mRNA abundance was observed (data not shown). Cells were harvested at different time points from 1 to 48 h. Total RNA was purified and subjected to Northern analysis for determining AtFer1 mRNA abundance.
abundance (Fig. 1A). In response to iron treatment, the AtFer1 steady state mRNA level increased 1 h after iron application, reached a maximum after 3 to 6 h of treatment, and decreased. This result is in accordance with those obtained with Arabidopsis plantlets (20, 21). The AtFer1 mRNA abundance was also increased after H$_2$O$_2$ treatment, but with a different time course; the maximum level of transcript was observed 12 h after H$_2$O$_2$ treatment, and AtFer1 mRNA was barely detectable after 24 h (Fig. 1A). AtFer1 mRNA abundance after co-treatment with both effectors was compared with the effect of iron or H$_2$O$_2$ applied alone. This result indicates that iron and H$_2$O$_2$ are acting in two independent and additive pathways leading to AtFer1 mRNA accumulation. However, addition of iron could cause the production of a certain amount of H$_2$O$_2$, potentially leading to a further AtFer1 mRNA accumulation. Such an hypothesis is unlikely because when cells were treated with catalase prior to iron addition, no change in AtFer1 mRNA accumulation was observed compared with catalase untreated cells (Fig. 1C). At the protein level, ferritin was accumulated after 24 h of iron treatment. It was also accumulated at the same time point after H$_2$O$_2$ treatment, but to a lower extend. Co-treatment with iron and H$_2$O$_2$ led to a ferritin protein accumulation to a level close to the one that was observed in response to iron treatment (Fig. 1B). Moreover, we have tested the effect of okadaic acid (OA) on AtFer1 mRNA abundance in response to iron excess or H$_2$O$_2$ treatments. It has to be reminded that OA has been shown to antagonize maize ZmFer1 gene expression both in response to iron excess and H$_2$O$_2$ treatment. When Arabidopsis

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Iron and H$_2$O$_2$ increase AtFer1 mRNA abundance by independent pathways. A, kinetic of AtFer1 mRNA abundance in response to iron and H$_2$O$_2$ treatments. Arabidopsis culture cells were treated with 300 μM iron-citrate or 5 mM H$_2$O$_2$, for different times. RNA was analyzed by Northern blotting and hybridized successively with the AtFer1 (upper panel) and EF1α (lower panel) probes. EF1α mRNA abundance was shown as loading control. B, effect of co-treatment with iron and H$_2$O$_2$ on mRNA and protein accumulation. For mRNA abundance determination, cells were treated for 6 h either with 5 mM H$_2$O$_2$ or 300 μM iron-citrate or co-treated with 5 mM H$_2$O$_2$, 300 μM iron-citrate. Untreated cells were used as control. Relative AtFer1 mRNA abundance (AtFer1/EF1α signal intensities ratio) was determined from three independent experiments. Bars correspond to the standard deviation. For AtFer1 protein detection, cells were treated for 24 h either with 5 mM H$_2$O$_2$, or 300 μM iron-citrate or co-treated with 5 mM H$_2$O$_2$, 300 μM iron-citrate. Ten-μg protein extracts were loaded for each lane. A polyclonal serum (1/20,000 dilution) raised against AtFer1p was used for immunodetection. A Coomassie Blue-stained gel is shown as loading control. C, effect of catalase on the iron- and H$_2$O$_2$-mediated expression of AtFer1. Arabidopsis cells were pre-treated, when indicated, with 140 units ml$^{-1}$ of catalase for 1 h. Either 300 μM iron-citrate or 5 mM H$_2$O$_2$ were then added to the culture medium. Cells were collected at different time points. Northern blot was performed as described above. D, effect of OA on the iron- and H$_2$O$_2$-mediated expression of AtFer1. Arabidopsis cells were pre-treated, where indicated, with 250 nM OA for 3 h. Either 300 μM iron-citrate or 5 mM H$_2$O$_2$ were then added to the culture medium. Cells were collected at different time points. Northern blot was performed as described in A.
cells were treated with OA, iron-induced AtFer1 mRNA abundance was decreased, whereas H2O2-induced mRNA abundance was increased (Fig. 1D). This indicates that a PP2A-type phosphatase is a positive regulator of the iron pathway and a negative regulator of the H2O2 pathway. This result enforces the hypothesis of independent pathways. Although we cannot rule out that reactive oxygen species other than H2O2 could be involved in the increase of AtFer1 mRNA abundance, it is more likely that the response is specific to H2O2 because catalase addition prior to H2O2 treatment abolished AtFer1 mRNA accumulation (Fig. 1C). Our main goal being to decipher the iron- and IDRS-dependent pathway, we further investigate only the response of AtFer1 gene to iron treatment.

An Iron-induced Plastidial NO Burst Precedes AtFer1 mRNA Accumulation—Several reports have involved nitric oxide in the control of iron homeostasis and in AtFer1 regulation (26, 27, 36–38). NO was shown to be involved in iron- and IDRS-dependent AtFer1 regulation (26, 27). SNP (a NO donor) induced an AtFer1 mRNA abundance increase and cPTIO (a NO scavenger) antagonized iron-induced AtFer1 mRNA accumulation (Fig. 1C). Our main goal being to decipher the iron- and IDRS-dependent pathway, we further investigate only the response of AtFer1 gene to iron treatment.

FIGURE 2. Iron treatment leads to a plastid-located NO burst. A, kinetic of NO accumulation in iron-treated cells. Cells were loaded with DAF-FM DA for 30 min, and 300 μM iron-citrate or 300 μM K2-citrate was added. The fluorescence of DAF-FM DA (excitation 495 nm, emission 515 nm) was visualized by confocal laser scanning at different time points. B, localization of NO production in plastids. In the same cells, chlorophyll autofluorescence and DAF-FM DA were visualized, and the two images were superimposed.

The next step was to examine further the origin of the NO production regulating AtFer1 expression. Cells were treated with animal NO synthase inhibitors prior to iron addition. A treatment with L-NMMA decreased AtFer1 mRNA abundance in response to iron treatment (Fig. 3A). The same decrease was obtained with Aω-nitro-L-arginine methyl ester (data not shown). We used both SNP and cPTIO as controls, and, as previously reported (26, 27), these compounds increased and
Regulation of Ferritin by NO and Protein Degradation

A. Fe-citrate

| Time (h) | ∅       | c-PTIO | L-NMMA | SNP |
|---------|---------|--------|--------|-----|
| 0       | 0       | 0      | 0      | 0   |
| 3       | 0       | 0      | 0      | 0   |
| 6       | 0       | 0      | 0      | 0   |

AtFer1

EF1α

B. Fe-citrate

| Time (h) | Col | nos1 | Col | g'4-3 |
|---------|-----|------|-----|-------|
| 0       | 0   | 0    | 0   | 0     |
| 3       | 0   | 0    | 0   | 0     |
| 6       | 0   | 0    | 0   | 0     |

AtFer1

EF1α

FIGURE 3. Implication of nitric oxide on iron-mediated AtFer1 expression. A, effect of NO scavenger, NO donor, and NOS inhibitors. Cells were pre-treated for 1 h with 1 mM c-PTIO or 5 mM l-NMMA prior to 300 μM iron-citrate addition. SNP (2.5 mM) was added without iron. RNA was extracted at different time points. Northern blot analysis was performed as described in the legend to Fig. 1. B, implication of NR and NOS1 in AtFer1 iron-mediated expression. Ten-day-old wild-type Arabidopsis (Columbia ecotype), nos1 and g'4-3 mutants plantlets, cultivated in vitro, were treated, where indicated, with 300 μM FeSO₄•6Na₂-Citrate for 3 and 6 h. RNA extracted from plantlets were analyzed by Northern blotting and hybridized successively with AtFer1 and EF1α probes.

decreased, respectively, AtFer1 mRNA abundance (Fig. 3A). The decrease observed with l-NMMA treatment suggests that a NO synthase activity could be required for the iron-dependent AtFer1 up-regulation. So far, two enzymes have been implicated in NO production in plants (39, 40): the nitrate reductase and the NO synthase NOS1. To determine whether these enzymes are involved in NO production leading to AtFer1 mRNA accumulation in response to iron excess, the loss of function mutants g'4-3 (30) and atnos1 (29) were used. The g'4-3 mutant is deficient in both nia1 and nia2 gene activities and displays only 0.5% of the wild-type shoot nitrate reductase (NR) activity. Arabidopsis cell lines of these two mutants are not available. Therefore, experiments were performed on plantlets grown under sterile conditions. In this plantlet system, AtFer1 response to iron treatment is not altered (20), and NO treatment led to an increase of AtFer1 mRNA abundance, in the same manner as in the cell culture system (data not shown). In the two mutants, the abundance of AtFer1 mRNA after iron application was similar to the one observed in wild-type plants (Fig. 3B). These results indicate that neither nitrate reductase nor NOS1 are involved in iron-dependent NO production leading to AtFer1 regulation.

AtFer1 Repressor Is Degraded by a 26 S Proteasome-dependent Pathway—The cis-acting element IDR5 has been shown to be involved in iron-dependent AtFer1 de-repression (18). This suggests that a repressor acts in the pathway, and that iron addition leads to a de-repression of AtFer1 transcription, rather than to activation (18, 27). This also suggests that iron treatment may lead to the inactivation or the degradation of a repressor. To test this latter hypothesis, and more specifically the involvement of a 26 S proteasome-dependent protein degradation, we used the specific 26 S proteasome inhibitor MG132. Cells were treated with MG132 prior to iron treatment. The repressor acts in the pathway, and that iron addition leads to a de-repression of AtFer1 transcription, rather than to activation (18, 27). This also suggests that iron treatment may lead to the inactivation or the degradation of a repressor. To test this latter hypothesis, and more specifically the involvement of a 26 S proteasome-dependent protein degradation, we used the specific 26 S proteasome inhibitor MG132. Cells were treated with MG132 prior to iron treatment. A general serine protease inhibitor, Pefabloc, was used in the same conditions as a control (Fig. 4A). Pefabloc did not affect the iron-dependent AtFer1 regulation, whereas MG132 treatment completely abolished the response. This result indicates that a protein, involved in the repression of AtFer1 transcription in low iron conditions, is ubiquitinated and degraded by a 26 S proteasome-dependent pathway after iron treatment. This factor was named repressor.

As protein degradation appears to be involved in AtFer1 de-repression, we have also checked whether protein synthesis
Regulation of Ferritin by NO and Protein Degradation

Upstream in the pathway. It can be hypothesized that degradation of some proteins could be required for NO production. Thus, altering the NO production by MG132 could also explain why AtFer1 mRNA does not accumulate in response to treatment with this proteasome inhibitor. To show that NO generation in response to iron treatment occurs independently of the proteasome, Arabidopsis cells were treated with iron and MG132, and NO production was monitored by DAF imaging. The fluorescence observed was similar to the one of control cells treated with iron in the absence of MG132, showing that iron-dependent NO production was not altered by MG132 (Fig. 5B).

**AtFer1 Repressor Is Not Bound to the IDRS**—As the IDRS cis-acting element has been shown to be involved in AtFer1 de-repression, it was tempting to postulate that the repressor could be a transcription factor bound to the IDRS in low iron conditions, and degraded upon iron addition. To test this hypothesis, we first checked whether the AtFer1 IDRS could bind nuclear factors. Nuclear extracts were prepared from untreated cells and from cells treated with iron (de-repression condition), or with MG132 (repression condition). A 32P radio-labeled DNA probe corresponding to a 200-bp region of the AtFer1 promoter sequence, and containing the IDRS (probe A; Fig. 6A), was incubated with nuclear proteins. Only one complex was observed by gel shift (Fig. 6B). To check the specificity of this complex, the binding reaction was performed with a 50-fold molar excess of different unlabelled DNA fragments. With DNA fragments containing the IDRS (probes A, B, and C, Fig. 6A), the signal corresponding to the complex was completely abolished, whereas it was not modified with probes that did not contain the IDRS sequence (probes D and E, Fig. 6A)

**DISCUSSION**

Our current knowledge on ferritin gene expression in plants is largely based on work on maize ZmFer genes (18, 19, 22, 25) and on the Arabidopsis AtFer1 gene (20, 21, 23, 27, 28). We used an Arabidopsis cell culture system to further characterize the pathway leading to AtFer1 de-repression after iron addition.

The AtFer1 mRNA level is enhanced by both iron and H2O2 treatments (Fig. 1A). This result is consistent with the studies on the maize ZmFer1 gene, which is orthologous to AtFer1 (22). As a highly reactive transition metal, iron may lead to oxidative stress. By sequestering free iron, the accumulation of ferritin may prevent oxidative damage. In maize, ZmFer1 mRNA abundance in response to iron treatment is antagonized by antioxidants like N-acetylcysteine and GSH, indicating that the iron effect on ferritin mRNA abundance is dependent of an oxidative step (22). Both iron- and H2O2-dependent ZmFer1 mRNA

---

**FIGURE 5.** Order of the molecular events in iron-mediated AtFer1 expression signaling pathway. **A**, effect of co-treatments on AtFer1 mRNA abundance. Cells were pre-treated, where indicated, with 250 nm OA or 50 μM MG132 for 3 and 1 h, respectively. Then, SNP (2.5 mM final concentration) was added into medium culture, and cells were collected 1 h later. Northern blot was performed as described in the legend to Fig. 1. **B**, effect of MG132 on the iron-mediated NO burst. Cells were loaded with DAF-FM DA for 30 min, and MG132 (final concentration 50 μM) or Me2SO (DMSO) (control) were added. Cells were incubated for 15 min. The fluorescence of DAF-FM DA (excitation 495 nm, emission 515 nm) was visualized by confocal laser scanning 20 min after iron-citrate addition.
Regulation of Ferritin by NO and Protein Degradation

A. 40 bp

B. Radiolabelled probe A

Regulation of ferritin by NO and protein degradation is sensitive to okadaic acid. This is consistent with the hypothesis that these inducers could act through the same oxidative pathway leading to an increased ZmFer1 mRNA abundance (22). In contrast to ZmFer1, OA strongly decreased AtFer1 mRNA abundance in response to iron (Fig. 1D). Furthermore, the amount of the AtFer1 transcript in response to H2O2 is increased after okadaic acid treatment. Thus, a PP2A-dependent pathway, and a repressor of the H2O2-dependent pathway. The two pathways appear therefore totally independent. This is in full agreement with the observation that the abundance of the AtFer1 mRNA in response to the addition of the two effectors at the same time is closed to the sum of the transcript abundance observed in response to each effector when applied alone (Fig. 1B). However, the iron-dependent AtFer1 mRNA increase in abundance has been previously shown to be antagonized by N-acetylcysteine treatment, revealing the involvement of an oxidative step in this response (20). This suggests that two different oxidative signals are involved in both iron- and H2O2-dependent pathways. At the protein level, we observed a higher amount of ferritin accumulated in response to iron treatment than in response to H2O2 treatment (Fig. 1B). This is consistent with previous data indicating that iron is required for ferritin protein stabilization (25).

In the present work, we have shown that iron addition leads to NO accumulation in the plastids (Fig. 2). NO is an essential growth regulator in plants, and serves as a signal in biotic and abiotic stress responses, programmed cell death, hormone responses, root and xylem development, flowering, and iron homeostasis (reviewed in Refs. 37 and 39–45). The inhibitory effect of 1-NMMA on AtFer1 expression in response to iron excess indicates that NO synthase activity is involved in the pathway (Fig. 3). In plant cells, the two enzymes so far reported to be implicated in NO synthesis are the NR and a nitric-oxide synthase (NOS1). NR is a cytosolic located enzyme, and NOS1 has recently been shown to be targeted to the mitochondria (46). Thus, none of these enzymes could produce NO in the plastids. Such a conclusion is consistent with the observation that AtFer1 expression in response to iron is not altered in the mutants g’4-3 and atnos1 (Fig. 3). Although there is so far no conclusive evidence for enzymatic NO production in the plastids, there are two indirect observations in favor of this hypothesis. First, an immunoreactive NOS protein has been detected in plastids (47). Second, the addition of the plant defense elicitor cryptogein on epidermal tobacco cells leads to a Nω-nitro-L-arginine methyl ester-sensitive NO production in the plastids. Such results indicate that NO synthase activity is probably present in the plastid, but the nature of the enzyme involved remains to be determined.

A NO scavenger, cPTIO, completely abolishes iron-dependent AtFer1 expression, clearly establishing that NO is a major element in this signal transduction pathway. In contrast to cPTIO, 1-NMMA application decreases AtFer1 response by only 50% when compared with untreated cells (based on relative AtFer1 mRNA abundance compared with EF1α; data not shown). Such a partial inhibition could be attributed to an incomplete action of this inhibitory compound. However, it cannot be excluded that both an enzymatic and a non-enzymatic (insensitive to the inhibitor used) pathway may lead to NO production in response to iron. Indeed, it is known that in plants, non-enzymatic NO production can arise from reactions between nitrite and various plant metabolites (50–52). Such a non-enzymatic NO production from nitrite has in particular been reported to occur at acidic pH in the apoplasm for example (52) and could explain the NO effects on germinating seeds (52–54). However, such a nitrite-dependent NO production is unlikely to occur in the stroma of plastids where the pH value is 7.0 or higher (55, 56).

Regardless the origin of the NO produced in the plastid in response to iron, this NO burst is an early event in the signal transduction pathway. This suggests that a retrograde signal, of unknown nature, could be produced in the plastid and lead to

**FIGURE 6. Specific binding of Arabidopsis nuclear protein(s) to the IDRS.**

A, localization of the DNA probes used for the gel shift experiments on AtFer1 promoter region. The putative regulatory elements indicated by boxes are those defined by Petit et al. (16). TATA, TATA box; B, mobility shift assays. Five μg of nuclear protein extracts from Arabidopsis cells untreated (−Fe), or treated for 3 h either with 300 μM iron-citrate (+Fe) or 1 h with 50 μM MG132 (+MG132) were incubated for 30 min at room temperature with radiolabeled probe A. For the competition assays, a 50-fold molar excess of unlabeled fragments was added in the reaction.

1B).
the transcription of the nuclear-encoded AtFer1 gene. Two major future prospects arise from this work and concern the identification of the site of action of NO in the pathway, and the nature of the retrograde signal. In animals, it is documented that the redox-related species of NO can have simultaneous effects on cellular iron metabolism and homeostasis via mechanisms that might involve S-nitrosylation (57), ligation of NO to iron-sulfur clusters (58, 59) or to heme-containing proteins (60, 61). NO and free iron may also form complexes with low molecular weight thiols like glutathione and cysteine (62–64). These dinitrosyl-iron complexes are relatively stable in contrast to the highly reactive free NO molecule (65, 66) and were shown to be potential NO carrier molecules in mammals (62). In plants, interactions of NO with hemes (67–69) or iron-sulfur clusters (70), and S-nitrosylation reactions (71) have been shown, and dinitrosyl-iron complexes have been detected (72). A post-translational modification of a plastidial protein by NO could be involved in the pathway leading to AtFer1 de-repression. Alternatively, a dinitrosyl-iron complex with glutathione, which has been shown to permeate quickly through membranes (73), could be a good candidate for a retrograde signal.

Plant ferritin mRNA accumulation can be promoted by ABA (25), H2O2 (Refs. 21 and 22, this work), and NO (Ref. 27, this work). Whether or not these inducers act in the same pathway has not been documented. Interestingly, by combining pharmacological, biochemical, and genetic approaches, it has recently been demonstrated that ABA-induced NO production via NR is required for ABA-induced H2O2-mediated stomatal closure (74). It is, however, unlikely that such a pathway occurs in the regulation of AtFer1 gene expression for the following reasons. First, we show that NO production in response to iron treatment is not mediated by NR (Fig. 3B). Second, among the ferritin gene families, only AtFer2 in Arabidopsis and ZmFer2 in maize have been reported to be regulated by ABA (19, 21). The AtFer1 gene is not regulated by ABA, and the ABA-regulated AtFer2 gene is not modulated by H2O2 (21).

A growing number of reports involve ubiquitination of positive or negative regulatory proteins, and their subsequent 26 S proteasome-dependent degradation as key steps of control within signaling pathways (75–77). Protein ubiquitination and subsequent degradation have been involved in light, auxin, ethylene, pathogen resistance, and more recently in phosphate starvation responses (78). The presence of a repressor in iron-mediated AtFer1 regulation of expression prompted us to examine whether inhibitors of the proteasome-dependent protein degradation could alter the iron response or not. Indeed, the use of MG132 dramatically decreases AtFer1 mRNA abundance. This result indicates that a protein acting in low iron conditions, called the repressor, is ubiquitinated after iron addition and subsequently degraded by the 26 S proteasome. This iron-triggered degradation leads to a de-repression of AtFer1 transcription and to the increase in abundance of the corresponding mRNA. Protein synthesis inhibition by cycloheximide perturbs such an increase, because at later time points of the kinetic (from 6 to 12 h) protein synthesis inhibition leads to a higher increased abundance of the AtFer1 mRNA compared with control cells untreated with cycloheximide (Fig. 4B). Therefore, it can be proposed that de novo synthesis of the repressor is necessary to decrease AtFer1 mRNA abundance about 6–9 h after iron addition. In fact, such a cycloheximide-promoted superinduction has been largely documented and can be attributed not only to a transcriptional de-repression (79, 80) but also to a decrease of mRNA degradation (79–82). Our results cannot discriminate between these two possibilities.

Mutagenesis of the IDR cis-element within the maize ZmFer1 or Arabidopsis AtFer1 promoter sequences leads to de-repression of the expression of reporter genes, in the absence of iron treatment (18). It can be therefore hypothesized that the repressor could bind to the IDR in low iron conditions and that iron treatment would promote its ubiquitination and degradation, resulting in AtFer1 gene expression. MG132 treatment of the cells would avoid the repressor degradation, maintain its binding to the IDR and repress AtFer1 gene expression, even after iron treatment. However, nuclear protein(s) from cells either treated with iron or MG132, or untreated bind equally to the IDR, without significant difference in size or intensity of the complex formed (Fig. 6). Therefore, the protein ubiquitinated and degraded in response to iron treatment, and defined as the repressor, is unlikely to be a trans-acting factor able to directly bind to the IDR in low iron conditions. This result is consistent with the observation that no significant difference in the intensity of the ZmFer1 IDR-protein complex was observed by gel shift experiments using nuclear extracts of iron-treated or untreated maize plants (18). Thus, it is proposed that the transcription factor(s) bound to the IDR is(are) essential but not sufficient for AtFer1 de-repression, and that the repressor acts upstream of this transcription factor.

It is worth noticing that in animal cells, one of the ferritin trans regulators involved in the translational repression of ferritin mRNAs in low iron conditions, namely IRP2, is regulated by NO and ubiquitin. After iron addition, IRP2 has been shown to be nitrosylated and subsequently ubiquitinated, and degraded by the proteasome, thus leading to ferritin mRNA translation (14, 15). Our work shows that NO and protein degradation via proteasome are also involved in the regulation of ferritin gene expression in plants. It is remarkable that molecular effectors of the response to iron excess, such as NO and ubiquitination, are conserved between the translational regulation of animal ferritin and the transcriptional regulation of plant ferritin.

Acknowledgments—We thank Dr. Nigel Crawford for providing the atnos1 mutant seeds. We thank Drs. Geneviève Conejero and Gaëlle Viennois for expertise in confocal microscopy. We thank Gabriel Kroauk, Karl Ravel, and Drs. Alain Pugin and Thierry Lagrange for helpful discussions.

REFERENCES
1. Guerinot, M. L., and Yi, Y. (1994) Plant Physiol. 104, 815–820
2. Noctor, G., and Foyer, C. H. (1998) Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 249–279
3. Harrison, P. M., and Arosio, P. (1996) Biochim. Biophys. Acta 1275, 161–203
4. Hentze, M. W., and Kuhn, L. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8175–8182

AUGUST 18, 2006•VOLUME 281•NUMBER 33
JOURNAL OF BIOLOGICAL CHEMISTRY 23587
