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RESEARCH PAPER

Ethylene and nitric oxide involvement in the up-regulation of key genes related to iron acquisition and homeostasis in *Arabidopsis*

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Abstract

In a previous work it was shown that ethylene participates in the up-regulation of several Fe acquisition genes of *Arabidopsis*, such as *AtFIT*, *AtFRO2*, and *AtIRT1*. In this work the relationship between ethylene and Fe-related genes in *Arabidopsis* has been looked at in more depth. Genes induced by Fe deficiency regulated by ethylene were searched for. For this, studies were conducted, using microarray analysis and reverse transcription-PCR (RT-PCR), to determine which of the genes up-regulated by Fe deficiency are simultaneously suppressed by two different ethylene inhibitors (cobalt and silver thiosulphate), assessing their regulation by ethylene in additional experiments. In a complementary experiment, it was determined that the Fe-related genes up-regulated by ethylene were also responsive to nitric oxide (NO). Further studies were performed to analyse whether Fe deficiency up-regulates the expression of genes involved in ethylene biosynthesis [*S*-adenosylmethionine synthetase, 1-aminocyclopropane-1-carboxylate (ACC) synthase, and ACC oxidase genes] and signalling (*AtETR1*, *AtCTR1*, *AtEIN2*, *AtEIN3*, *AtEIL1*, and *AtEIL3*). The results obtained show that both ethylene and NO are involved in the up-regulation of many important Fe-regulated genes of *Arabidopsis*, such as *AtFIT*, *AtbHLH38*, *AtbHLH39*, *AtFRO2*, *AtIRT1*, *AtNAS1*, *AtNAS2*, *AtFRD3*, *AtMYB72*, and others. In addition, the results show that Fe deficiency up-regulates genes involved in both ethylene synthesis (*AtSAM1*, *AtSAM2*, *AtACS4*, *AtACS6*, *AtACS9*, *AtACO1*, and *AtACO2*) and signalling (*AtETR1*, *AtCTR1*, *AtEIN2*, *AtEIN3*, *AtEIL1*, and *AtEIL3*) in the roots.

Key words: *Arabidopsis*, bHLH38, bHLH39, ethylene, FIT, iron, iron acquisition genes, iron deficiency, nitric oxide.

Introduction

In dicots and non-grass monocots (Strategy I plants), several Fe-regulated proteins, such as ferric reductases, Fe transporters, and H^+-ATPases, play key roles in root Fe uptake (see reviews by Curie and Briat, 2003; Hell and Sthepan, 2003; Walker and Connolly, 2008). In recent years, several genes that encode these proteins have been identified, such as the ferric reductase gene *AtFRO2* (Robinson *et al.*, 1999), the Fe transporter gene *AtIRT1* (Eide *et al.*, 1996), and the H^+-ATPase gene *AtAHA7* of *Arabidopsis* (Colangelo and Guerinot, 2004). Homologues of these genes have also been identified in other Strategy I plant species, such as tomato, pea, and cucumber (Eckhardt *et al.*, 2001; Waters *et al.*, 2002; Li *et al.*, 2004; Santi *et al.*, 2005; Waters *et al.*, 2007).

The regulation of these Fe acquisition genes is not totally known, but in recent years several transcription factors that participate in their activation have been found. Two of them are the tomato FER protein, identified as a basic helix–loop–helix (bHLH) transcription factor (Ling *et al.*, 2002), and the *Arabidopsis* FIT protein (previously named

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; EDDHA, *N*,*N*′-ethylenebis[2-(2-hydroxyphenyl)-glycine]; ferrozine, 3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonic acid)-1,2,4-triazine; GSNO, S-nitrosoglutathione; STS, silver thiosulphate.

bHLH29, FIT1, or FRU), a homologue of the tomato FER protein (Bauer *et al.*, 2007). Recently, other bHLH transcription factors, such as bHLH38 and bHLH39, have been implicated in the activation of the above-mentioned Fe acquisition genes (Vorwieger *et al.*, 2007; Walker and Connolly, 2008; Yuan *et al.*, 2008). Yuan *et al.* (2008) have shown that the overexpression of *AtFIT* with either *AtbHLH38* or *AtbHLH39* converted the expression of *AtFRO2* and *AtIRT1* from Fe deficiency induced to constitutive. These authors then suggest that the transcription of *AtFRO2* and *AtIRT1* is directly regulated by a complex of FIT/bHLH38 or FIT/bHLH39. *SlFER*, *AtFIT*, *AtbHLH38*, and *AtbHLH39* expression is induced in roots in response to Fe deficiency (Bauer *et al.*, 2004; Colangelo and Guerinot, 2004; Jakoby *et al.*, 2004; Brumbarova and Bauer, 2005; Vorwieger *et al.*, 2007; Yuan *et al.*, 2008). Besides the above-mentioned genes, other genes related to Fe nutrition, such as *AtFRD3* (encoding a transporter involved in the loading of citrate into the xylem; Durrett *et al.*, 2007) and *AtNAS* (involved in the biosynthesis of nicotianamine; Suzuki *et al.*, 1999, 2006; Klatte *et al.*, 2009) are also up-regulated under Fe deficiency.

In a previous work, Lucena *et al.* (2006) showed that ethylene could regulate *FRO* and *IRT* gene expression by affecting *AtFIT* (or *SlFER*) gene activity. These authors found that the treatment of Fe-deficient tomato or *Arabidopsis* plants with inhibitors of ethylene synthesis or action greatly decreased the *SlFER* or *AtFIT* mRNA accumulation, while treatment of tomato or *Arabidopsis* plants, grown in low Fe conditions, with 1-aminocyclopropane-1-carboxylate (ACC; an ethylene precursor) enhanced it. These authors have proposed a model in which ethylene acts as an activator of the *SlFER* (or *AtFIT*) gene, and consequently of the *FRO* and *IRT* genes, while Fe (probably phloem Fe) acts as an inhibitor (Lucena *et al.*, 2006). Later on, Graziano and Lamattina (2007) showed that nitric oxide (NO) can also up-regulate several Fe acquisition genes in tomato, such as *SlFER*, *SlFRO1*, and *SlIRT1*, in a manner very similar to ethylene. This raises the question as to whether NO acts downstream of ethylene, or ethylene downstream of NO, or if both act in conjunction.

Besides physiological responses, Strategy I plants also develop morphological responses to Fe deficiency, such as the formation of subapical root hairs and root epidermal transfer cells (Romera and Alcántara, 2004). These morphological responses are also probably regulated by ethylene, since both of them are induced in Fe-sufficient Strategy I plants upon ACC treatment (Romera and Alcántara, 2004). However, their induction in tomato plants does not depend on the FER transcription factor since both subapical root hairs and transfer cells are also induced in the tomato *fer* mutant upon ACC treatment (Schmidt *et al.*, 2000*a*; Romera and Alcántara, 2004).

Ethylene production increases under Fe deficiency in the roots of several Strategy I plants (Romera *et al.*, 1999; Waters and Blevins, 2000; Li and Li, 2004; Molassiotis *et al.*, 2005; Zuchi *et al.*, 2009). Ethylene production also increases under other nutrient deficiencies, such as of phosphorus or potassium (Lynch and Brown, 1997; Gilbert *et al.*, 2000; Shin and Schachtman, 2004; Jung *et al.*, 2009). In some of these nutrient deficiencies, ethylene has been shown to be involved in the regulation of morphological and physiological responses, such as the promotion of root hairs and the enhancement of transporters, developed by the plants to cope with these nutrient constraints (Borch *et al.*, 1999; Jung *et al.*, 2009).

One of the objectives of this work was to study the involvement of ethylene in the expression of genes of *Arabidopsis* up-regulated by Fe deficiency, besides those where its involvement has already been reported (Lucena *et al.*, 2006). For this, using microarray analysis and reverse transcription-PCR (RT-PCR), experiments were conducted to determine which of the genes up-regulated by Fe deficiency in the roots are simultaneously suppressed by two different ethylene inhibitors [cobalt (Co) and silver thiosulphate (STS)], assessing their probable regulation by ethylene in additional experiments. In a complementary experiment, tests were carried out to determine whether the Fe-related genes responsive to ethylene are also responsive to NO. Another objective of this work was to analyse whether Fe deficiency up-regulates the expression of genes involved in ethylene biosynthesis (*S*-adenosylmethionine (SAM) synthetase, ACC synthase, and ACC oxidase genes) and signalling (*AtETR1*, *AtCTR1*, *AtEIN2*, *AtEIN3*, *AtEIL1*, and *AtEIL3*).

Materials and methods

Plant materials, growth conditions, and treatments

Seeds of the *Arabidopsis thaliana* (L.) Heynh ecotype 'Columbia' and the ethylene-insensitive mutant *ein2-1* were germinated in black peat. When plants were 30 d old, they were inserted in plastic lids and held in the holes of a thin polyurethane raft floating on aerated nutrient solution. After 10–15 d in this hydroponic system, the plants were individually transferred to 70 ml plastic vessels containing continuously aerated nutrient solution with the different treatments. The plastic vessels were grouped in arrays containing 12 plant replications. Plants were grown in a growth chamber at 22 °C day/20 °C night temperatures, with relative humidity between 50% and 70%, and an 8 h photoperiod (to postpone flowering) at a photosynthetic irradiance of 300 μmol m^{-2} s^{-1} provided by fluorescent tubes (Sylvania Cool White VHO).

The nutrient solution (without Fe) had the following composition: 2 mM $Ca(NO_3)_2$, 0.75 mM K_2SO_4, 0.65 mM $MgSO_4$, 0.5 mM KH_2PO_4, 50 μM KCl, 10 μM H_3BO_3, 1 μM $MnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM $ZnSO_4$, 0.05 μM $(NH_4)_6Mo_7O_{24}$. Fe-EDDHA was added at different concentrations depending on the experiments. The pH was adjusted to 6.0 with 0.1 N KOH.

The treatments imposed were: +Fe40, +Fe10, nutrient solution with 40 μM or 10 μM Fe-EDDHA; +Fe+ethylene, the same as +Fe treatment but with ethylene addition during the last 24 h; +Fe+GSNO, the same as +Fe treatment but with *S*-nitrosoglutathione (GSNO) addition during the last 24 h; –Fe, nutrient solution without Fe (24 h); and –Fe+Co or –Fe+STS, –Fe treatment with $CoSO_4$ or STS added (24 h). Stock solution of GSNO was prepared by reacting equimolar (200 mM) concentrations of reduced glutathione and $NaNO_2$ in 0.1 M HCl. A stock solution of STS was prepared as previously described (Romera and Alcántara, 1994). After treatments, root ferric reductase activity

was determined as described below. Finally, the roots were collected and kept at –80 °C to analyse the mRNA later. Each treatment consists of six plant replications and, when appropriate, the results are presented as means ±SE.

For the treatments with ethylene, plants grown in nutrient solution with 10 μM Fe-EDDHA were treated with ethylene during the last 24 h as follows. A 6 ml aliquot of pure ethylene was injected into a 250 ml glass container sealed with a septum. Then, by means of a rubber tube adjusted with two syringes in both ends, one needle was inserted in the septum of the container and the other one in the aeration system (Fig. 1). Before and after the experiment, the ethylene content of the glass container was determined, showing that only ~1 ml of the initial 6 ml was used. This method, in contrast to other methods, allows the application of ethylene directly to the root.

Ferric reductase activity determination

Intact plants were pre-treated for 30 min in plastic vessels with 50–70 ml of a nutrient solution without micronutrients, pH 5.5, and then placed into 50–70 ml of an Fe(III) reduction assay solution for 30 min. This assay solution consisted of nutrient solution without micronutrients, 100 μM Fe (III)-EDTA, and 300 μM ferrozine, pH 5.0 (adjusted with 0.1 N KOH). The environmental conditions during the measurement of Fe(III) reduction were the same as the growth conditions described above. The ferric reductase activity was determined spectrophotometrically by measuring the absorbance (562 nm) of the Fe(II)–ferrozine complex and by using an extinction coefficient of 29 800 M^{-1} cm^{-1}. After the reduction assay, roots were excised and weighed, and the results were expressed on a root fresh weight basis.

RT-PCR analysis

Roots were ground to a fine powder using a mortar and pestle in liquid nitrogen. Total RNA was extracted using the Tri Reagent solution (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. M-MLV reverse transcriptase (Promega, Madison, WI, USA) was used to generate cDNA with 3 μg of total RNA from roots as the template and random hexamers as primers. Negative controls included all reaction components except M-MLV enzyme. One-tenth of each reverse transcription reaction was used as PCR template. *AtFRO2, AtIRT1*, and *AtFIT* cDNA were amplified with the primers described in Lucena *et al.* (2006). Oligonucleotides designed by Peng *et al.* (2005) were used to amplify *AtACS4*, *AtACS6*, and *AtACS9* cDNA. *AtBHLH38* and *AtBHLH39* cDNA were amplified with the primers designed by Vorwieger *et al.* (2007). *AtETR1* cDNA was amplified with the primers described in Wang *et al.* (2003). Oligonucleotides designed by Achard *et al.* (2003) were used to amplify *AtCTR1*. *AtEBP* cDNA was amplified with the primers described in Bürstenbinder *et al.* (2007). Pairs of primers were designed to amplify the other genes studied (Table 1). 18S cDNA was amplified using the QuantumRNA Universal 18S Standards primer set (Ambion, Austin, TX, USA) as internal control. The thermalcycler program was one initial cycle of 94 °C for 5 min; followed by cycles of 94 °C for 45 s; 55 °C for 45 s;

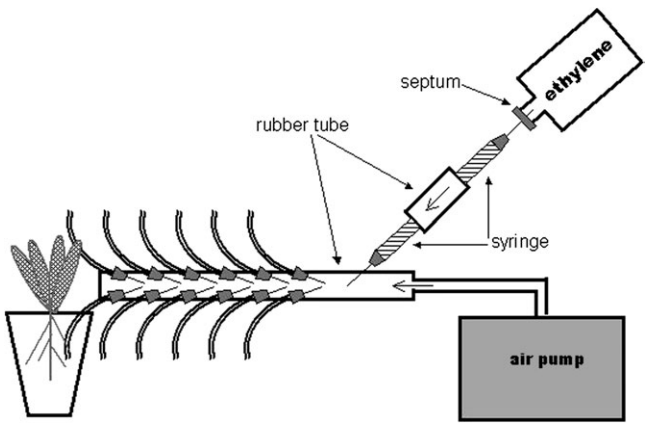

Fig. 1. System used for the application of ethylene to plants. For details, see Materials and methods.

Table 1. Primers designed for this study

Purpose	Sequence (5′–3′)
AtNAS1 forward	CAATTTTGGGATCTTTACAAGAAGA
AtNAS1 reverse	GTTGGATGATAGATGGTCAAGAGTT
AtNAS2 forward	ATTTAGAGCAACACTTTTCAGCAAT
AtNAS2 reverse	TGATAAATGGTTAACACCTCAAACC
AtCCCL1 forward	GAGAGCAGAGAAGGAGGAAGTG
AtCCCL1 reverse	GTAAGAGCCATAGCCATCCAAC
AtCCCL2 forward	TTTACCATGGAATCACACAACG
AtCCCL2 reverse	TAACCATCCTCCAATCAAAACC
AtCCCL3 forward	CAGATGGAGAGAGACAGTGTGG
AtCCCL3 reverse	ACACCAAAATCCACCGTAAAAC
AtFRD3 forward	CCAATCGCTTCTCTGATTGATAC
AtFRD3 reverse	GCCTTGCATAGCAAGAGATAGAA
AtAAT forward	CTGTCTTTGCTCCATTGACTTG
AtAAT reverse	GAAGTAAGTGGTGGAGCCGTAG
AtMYB72 forward	AAAACTGGAGATCTCTTCCCAAG
AtMYB72 reverse	TGAAGAAGAGAGTCTAGCGGAAA
AteIF-2B forward	AGCTATTGCTGCTGCTCTTTCT
AteIF-2B reverse	TTTCTCATGCACCAACTCAAAC
At2OGFe forward	ACCTGAAGGAAGTGAGTCCAAG
At2OGFe reverse	CACAGAGACTCTTGATCCGATG
AtCYP71B5 forward	ATCGAGGAAGCATTCAGATTACA
AtCYP71B5 reverse	AGATAACATGAAACATGCGGATT
AtBGlu42 forward	AGATTGAAGGAGGTTGGAATGA
AtBGlu42 reverse	GAACCTGATGATGTGAGACCAA
AtMTK forward	TTTACGCCGTTAAACGAGAAGT
AtMTK reverse	ATATGGGTCCGAAAACACAACT
At5g55620 forward	TTCAAACCTTCGTTTTCTTTCC
At5g55620 reverse	TACTTCAAAACATCCCCACACA
AtSAM1 forward	GACACCTGTCGCGCCATTGGAT
AtSAM1 reverse	CGTGAGCTCCCCATCCACCGTA
AtSAM2 forward	GTTAAGACCAGATGGCAAGACC
AtSAM2 reverse	TCCTCCTCTCTTCAAGTCCAAG
AtACS11 forward	ACAGCTGGATCAACCTCGGCT
At ACS11 reverse	GTCGCGGCTGACACCACTTTCT
AtACO1 forward	TGTCAGATCCCAAACATTTCAG
AtACO1 reverse	AAGAGCTTTGGAGCTGGAGATA
AtACO2 forward	CAAGCTCAATGGGGAAGAGA
AtACO2 reverse	GTGTGGGCCCTAAGACCTTT
AtACO3 forward	ACGACTTTTATCTCGACGAAGACAGGA
AtACO3 reverse	TGCTTCGTGTATTCGATCACCGCAC
AtEIN2 forward	CAACTTGCGAAAGAAAGAGGAT
AtEIN2 reverse	GGAGCTGTACTGCAAGGAGAAT
AtEIN3 forward	CAAGATGGGATCTTGAAGTATATGTTGAA
AtEIN3 reverse	CAGTCAAAACGCCGACTTTCCACGCC
AtEIL1 forward	ATCGTAATGGTCCAGCTGCTAT
AtEIL1 reverse	ACTCCTTTCTCCAAAGGAAACC
AtEIL3 forward	TGTGATCCTCCTCAAAGGAAGT
AtEIL3 reverse	ATGGCTGTTGGTTGTTCTTTTT
AtCHIT-B forward	ATGAAACTACAGGTGGATGGGCTACA
AtCHIT-B reverse	GGAAATAAAATCGCAACATAAACAGTG

72 °C for 1 min, with 27–30 cycles, all followed by a final 72 °C elongation cycle of 7 min.

Microarray analysis

cDNA was synthesized from 4 μg of total RNA using one-cycle target labelling and control reagents (Affymetrix, Santa Clara, CA, USA) to produce biotin-labelled cRNA. The cRNA preparation (15 μg) was fragmented at 94 °C for 35 min into segments 35–200 bases in length. If the quality control was correct, then 10 μg of fragmented cRNA was hybridized to the *Arabidopsis* ATH1 Genome array (Affymetrix), containing 22 500 transcript variants from 24 000 well-characterized *A. thaliana* genes. Three biological replicates for each condition were independently hybridized. Each microarray was scanned at 2.5 μm resolution in a GeneChip® Scanner 3000 7G System (Affymetrix). Data analyses were performed using GeneChip Operating Software (GCOS).

The robust multiarray analysis (RMA) algorithm was used for background correction, normalization, and summarization of expression levels (Irizarry *et al.*, 2003). Next, differential expression analysis was performed with the Bayes *t*-statistics from the linear models for Microarray data (limma). *P*-values were corrected for multiple testing using the Benjamini–Hochberg's method (false discovery rate) (Benjamini and Hochberg, 1995; Reiner *et al.*, 2003). Statistical analysis and graphical visualization of data were performed with the interactive tool FIESTA (http://bioinfogp.cnb.csic.es/tools/FIESTA). Some information about the genes studied have been obtained from the TAIR homepage.

Search for ethylene-responsive elements (EREs) in the promoters of IDED genes

The determination of EREs in the promoters of IDED genes was performed using the PLACE (Plant Cis-acting Regulatory DNA Elements) database (http://www.dna.affrc.go.jp/PLACE/); several types of known EREs were obtained from this database. The sequences of the promoter regions of the tested genes were obtained from the GenBank database. The frequency of the regulatory motifs in the 5′ upstream sequences of the *Arabidopsis* genes was calculated with the Patmach tool from the TAIR web site. *P*-values for the differences between observed and expected motif frequency due to chance were calculated using a binomial test.

Results

Identification of Fe deficiency-induced genes regulated by ethylene

Fe deficiency in roots induces a suite of genes, including those involved in Fe acquisition and homeostasis (Thimm *et al.*, 2001; Buckhout and Thimm, 2003; Colangelo and Guerinot, 2004; Walker and Connolly, 2008; Buckhout *et al.*, 2009). In a previous work, it was shown that the up-regulated expression of the Fe deficiency-induced genes *AtFIT*, *AtIRT1*, and *AtFRO2* was suppressed by ethylene inhibitors (Lucena *et al.*, 2006). Similarly, these three genes were up-regulated by ACC (an ethylene precursor) under low Fe conditions (Lucena *et al.*, 2006).

In this work, a DNA microarray analysis was performed to identify more genes up-regulated by Fe deficiency whose induction by Fe deficiency would depend, at least partly, on ethylene [iron deficiency-induced ethylene-dependent (IDED) genes]. Root RNA samples from Fe-sufficient plants were compared with those from Fe-deficient plants, and these latter were compared with those from Fe-deficient plants supplemented with the ethylene inhibitors Co or STS.

Microarray analysis revealed that 116 genes were at least 2-fold up-regulated in *Arabidopsis* roots in response to Fe deficiency. Among these 116 genes, there are genes related to Fe acquisition and homeostasis (see Supplementary Table S1 available at *JXB* online), which have been described as induced by Fe deficiency in other microarray analyses (Buckhout and Thimm, 2003; Colangelo and Guerinot, 2004; Buckhout *et al.*, 2009). The presence of Co in the nutrient solution negatively affected the Fe deficiency induction of 21 of these genes. Similarly, STS reduced the Fe deficiency induction of 24 of these genes. In order to increase the chance of selecting genes whose Fe deficiency induction was truly dependent on ethylene (IDED genes), the search was focused on 16 genes whose expression level under Fe deficiency was significantly reduced by both Co and STS.

Among these 16 IDED genes, there are iron and amino acid transport-related genes, transcription and translation factors, and genes for metabolic enzymes (Table 2). Some genes not listed in Table 2, such as those for the ferric reductase *AtFRO2*, and the transcription factors *AtbHLH38* and *AtbHLH39*, have also been identified as IDED genes. The *AtFRO2* and *AtbHLH38* genes are not included in the ATH1 chip but their regulation by the ethylene precursor ACC was shown in previous works (Lucena *et al.*, 2006; Romera, 2008). *AtbHLH39* is included in the ATH1 chip but it was not selected, at first, because it did not fulfil the statistical requirements used ($P < 0.05$). However, its up-regulation by ACC has already been reported by Romera (2008). The role of ethylene in the regulation of the 19 selected genes has been further confirmed by RT-PCR (Fig. 2). For this, experiments were performed to test whether ethylene inhibitors block the up-regulated expression of the IDED genes in Fe-deficient plants. In addition, the ability of ethylene itself (and not ACC) to induce the expression of the selected IDED genes was tested. As shown in Fig. 2a, ethylene inhibitors (Co and STS) negatively affect the expression of all the IDED genes. On the other hand, transcripts from all these genes were more abundant in roots of Fe-sufficient plants treated with ethylene than in the untreated roos (Fig. 2b). Besides inducing IDED genes, ethylene treatment also induced the expression of some known ethylene-inducible genes, such as *AtCHIT-B* (chitinase) and *AtEBP* (ethylene-responsive element-binding protein, also known as *ERF72*) (Broglie *et al.*, 1986; Büttner and Singh, 1997; Bürstenbinder *et al.*, 2007) (Fig. 2b), and the formation of subapical root hairs (Fig. 4).

Genes related to Fe transport. The group of IDED genes functionally related to Fe transport is headed by *AtIRT1* (Table 2), the gene encoding the major transporter for Fe uptake from the soil (Eide *et al.*, 1996; Vert *et al.*, 2002). Ethylene (ACC) regulation of *IRT1* has been reported in previous papers (Lucena *et al.*, 2006, 2007).

Four genes for nicotianamine synthase (NAS) have been found in the genome of *Arabidopsis* (Suzuki *et al.*, 1999;

Table 2. Summary of microarray data

Genes listed on this table meet the following requirements. First, they are iron deficiency-induced genes (fold change >2 on Fe-depleted versus Fe-replete medium, except for translation factor At2g05830). Secondly, their induction on Fe-depleted medium was negatively affected by both ethylene inhibitors, Co and STS (fold change less than –2 on Fe-depleted medium containing Co or STS versus Fe-depleted medium without additions, except for translation factor At2g05830 and iron transporter IRT1). Translation factor At2g05830 and iron transporter IRT1 did not meet the fold change selected value range but were very close to it. Data in this table represent the average of three biological replicates and were found to be statistically significant (P <0.05). Within each group, genes are ordered by their response to iron deficiency.

Representative public ID	–Fe versus +Fe, fold change	–Fe+Co versus –Fe, fold change	–Fe+STS versus –Fe, fold change	Annotation
Iron transport				
At4g19690	27.3	–1.4	–1.6	IRT1, Fe(II) transporter
At5g56080	9.0	–7.6	–8.0	NAS2, nicotianamine synthase 2
At5g04950	7.5	–3.7	–3.7	NAS1, nicotianamine synthase 1
At3g25190	7.4	–13	–7.3	CCCL1, CCC1-like protein, putative metal transporter
At1g21140	6.2	–8.1	–4.6	CCCL2, CCC1-like protein, putative metal transporter
At1g76800	4.8	–8.5	–6.7	CCCL3, CCC1-like protein, putative metal transporter
At3g08040	4.1	–4.5	–4.0	FRD3, MATE efflux family protein
Amino acid transport				
At5g38820	3.7	–2.0	–3.1	AAT, amino acid transporter family protein
Transcription factor				
At2g28160	2.8	–2.2	–2.6	FIT, Fe-deficiency induced transcription factor 1
At1g56160	2.2	–2.1	–2.6	MYB72, myb family transcription factor
Translation factor				
At2g05830	1.8	–1.8	–2.1	eIF-2B, putative eukaryotic translation initiation factor 2B family protein
Metabolism				
At3g12900	24.9	–8.4	–6.8	2OGFe, 2OG-Fe(II) oxygenase family protein
At3g53280	3.5	–3.4	–3.6	CYP71B5, cytochrome P450 71B5
At5g36890	3.4	–2.9	–3.3	BGLU42, glycosyl hydrolase family 1 protein
At1g49820	2.4	–2.3	–2.9	MTK, 5-methylthioribose kinase
Unknown				
At5g55620	2.1	–2.1	–2.4	Expressed protein

Weber *et al.*, 2004; Klatte *et al.*, 2009), and samples of their sequences are included in the ATH1 chip used in this work, although it should be noted that *AtNAS3* is not expressed in roots (Suzuki *et al.*, 1999; Weber *et al* 2004; Klatte *et al.*, 2009). Two of the NAS genes, *AtNAS1* and *AtNAS2*, were identified as IDED genes and included in the group of genes related to Fe transport (Table 2), since NA is considered a chelator of Fe^{2+}, involved in its internal transport (Scholz *et al.*, 1992; Suzuki *et al.*, 1999; Pich *et al.*, 2001).

Three genes with sequence similarity to nodulins, *AtCCCL1*, *AtCCCL2*, and *AtCCCL3*, were also identified as IDED genes (Table 2). All of them encode putative membrane proteins similar to the yeast CCC1, which is involved in the transport of Fe^{2+} from the cytosol to the vacuole for storage (Li *et al.*, 2001).

AtFRD3 is another gene whose full induction by Fe deficiency seems to require ethylene (Table 2, Fig. 2). *AtFRD3* encodes a protein of the MATE (multidrug and toxin efflux) family responsible for the loading into the xylem of citrate, an Fe chelator, which is essential for the correct distribution of Fe throughout the plant tissues (Durrett *et al.*, 2007). Loss-of-function mutation *frd3* (formerly *man1*) causes severe alteration of Fe homeostasis, characterized by constitutive Fe deficiency responses and excessive Fe accumulation (Rogers and Guerinot, 2002).

Trancription factors. IDED transcription factors identified so far include FIT (bHLH29), bHLH38, bHLH39, and MYB72 (Table 2, Fig. 2). FIT, bHLH38, and bHLH39 are transcription factors involved in the activation of many Fe acquisition genes (Colangelo and Guerinot, 2004; Vorwieger *et al.*, 2007; Walker and Connolly, 2008; Yuan *et al.*, 2008). All of them (*AtFIT*, *AtbHLH38*, and *AtbHLH39*) are induced by ACC in *Arabidopsis* plants grown in low Fe conditions (Lucena *et al.*, 2006; Romera, 2008). Although the present microarray analysis only detected *AtFIT* and failed to detect *AtbHLH38* (it is not present in the ATH1 chip) and *AtbHLH39*, RT-PCR analysis showed that all three genes respond positively to ethylene (Fig. 2b) and that their response to Fe deficiency was diminished by both Co and STS (Fig. 2a).

MYB72 was previously identified as an Fe deficiency-induced transcription factor partially regulated by FIT (Colangelo and Guerinot, 2004). *AtMYB72* expression has been shown to depend on ethylene in this study (Table 2,

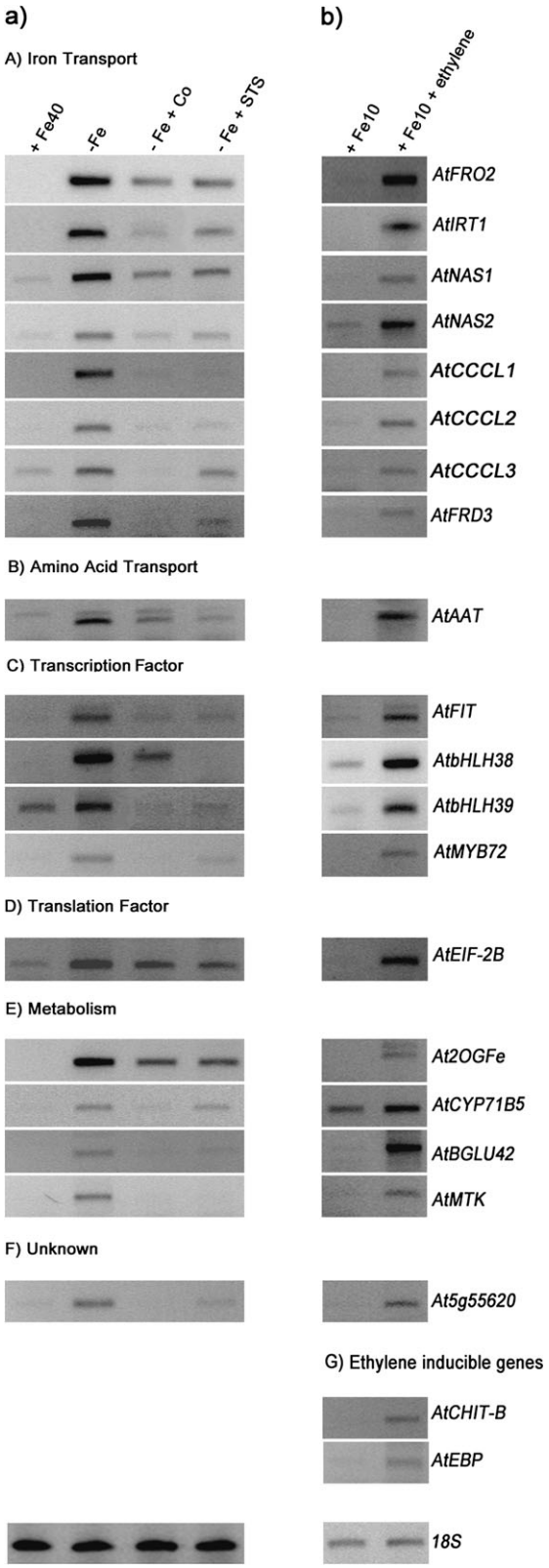

Fig. 2. (a) Effects of Fe deficiency and ethylene inhibitors on the expression of the selected genes. Plants grown in nutrient solution with 10 μM Fe were transferred for 24 h to nutrient solution with 40 μM Fe (+Fe40), without Fe (–Fe), without Fe supplemented with 50 μM Co (–Fe+Co), or without Fe supplemented with 200 μM STS (–Fe+STS). (b) Effect of ethylene treatment on the expression of the selected genes. Plants were grown in nutrient solution with 10 μM Fe (+Fe10) and half of them were treated during the last 24 h with ethylene (+Fe10+ethylene). RT-PCR was performed using total RNA from roots as template and gene-specific primers to amplify partial cDNAs from the genes indicated in the figure. *AtCHIT-B* and *AtEBP* are known ethylene-induced genes and were amplified as positive controls. 18S cDNA was amplified as an internal control.

Fig. 2). The *myb72* loss-of-function mutation confers sensitivity to Fe-deficient conditions (Van de Mortel *et al.*, 2008). MYB72 is also involved in induced systemic resistance (Van der Ent *et al.*, 2008; Segarra *et al.*, 2009).

Metabolic enzymes. Several genes encoding metabolic enzymes have also been identified as IDED genes (Table 2). At3g12900 encodes a protein of the 2OG-Fe(II) oxygenase family (2OGFe) whose members typically catalyse the 2-oxoglutarate- and Fe(II)-dependent oxidation of an organic substrate using a dioxygen molecule. In plants, enzymes of this family catalyse hydroxylation and desaturation steps in the synthesis of gibberellins, anthocyanidins, and flavones. The ethylene-producing enzyme ACC oxidase also belongs to this family (Aravind and Koonin, 2001).

At3g53280, another member of this group, encodes CYP71B5, a protein of the cytochrome P450 monooxygenase family whose members participate in the biosynthesis of a variety of compounds, such as hormones, defensive metabolites, fatty acids, and lignin. At5g36890 encodes BGLU42, a β-glucosidase belonging to the glycosyl hydrolase family 1 (GH1) from *Arabidopsis*. This family contains 48 members which are believed to play important roles in diverse processes including response to stress, lignification, phytohormone activation, and cell wall remodelling (Xu *et al.*, 2004; Opassiri *et al.*, 2006).

Last in this group is *AtMTK*, the single gene encoding *Arabidopsis* 5-methylthioribose kinase, an enzyme of the 'Yang cycle' that allows methionine recycling for ethylene synthesis and is essential for sustained ethylene production (Sauter *et al.*, 2004; Bürstenbinder *et al.*, 2007).

Others genes. Other IDED genes identified in this work are At5g38820, encoding an amino acid transporter (AtAAT); At2g05830, encoding a putative eukaryotic translation initiation factor from the 2B family (AteIF-2B); and At5g55620, coding for an unknown expressed protein (Table 2).

Occurrence of ethylene- and FIT-responsive elements in the promoters of IDED genes

The occurrence of EREs in the promoters of the selected IDED genes was investigated as additional evidence of their ethylene regulation. An 8 bp AWTTCAAA motif has been identified as an ERE in the promoter region of several ethylene-induced genes (Montgomery *et al.*, 1993; Itzhaki *et al.*, 1994; Tapia *et al.*, 2005). Fourteen out of 19 IDED genes identified in this study contained at least one AWTTCAAA motif and, in most of them, it is located in the 1000 bp of sequence upstream of the transcription

initiation site (Fig. 3). Exceptions are *AtFRO2*, *AtAAT*, *AteIF-2B*, *AtbHLH39*, and *AtBGLU42*. Nevertheless, *AtbHLH39* and *AtBGLU42* promoters contain the GCCGCC motif, which is a target sequence for the ethylene-responsive element-binding factor (ERF) transcription factor family (Chakravarthy *et al.*, 2003; Fig. 3).

The number of AWTTCAAA motifs found in the promoters of the 19 IDED genes (1000 bp upstream sequences) is 14, being higher than expected (8.76), according to the frequency of this motif in equivalent regions of the *Arabidopsis* genome as calculated with the Patmach tool (TAIR web page). This difference was statistically significant (binomial test P-value=0.003), suggesting an over-representation of this ERE in the IDED genes.

The bHLH transcription factor FIT is the main switcher of Fe deficiency responses, controlling the expression of a great number of Fe acquisition and homeostasis genes. In fact, most of the genes identified in this work as IDED genes have been previously reported as requiring FIT in order to be fully expressed (Colangelo and Guerinot, 2004). The bHLH transcription factor recognition sequence CANNTG (E-box) has been proposed as a possible target for FIT regulation (Colangelo and Guerinot, 2004). Multiple copies of this putative FIT responsive element were found in the promoters of the IDED genes identified in this study (Fig. 3). However, in spite of its abundance in the promoters of the IDED genes, the CANNTG motif was not found to be over-represented when compared with its occurrence in equivalent regions of the *Arabidopsis* genome (Patmach tool, TAIR web page). A high frequency of CANNTG, but not a statistically significant over-representation, has also been previously reported for a wider group of FIT-regulated genes (Colangelo and Guerinot, 2004) in which many of the IDED genes were included.

In summary, the occurrence of the above-mentioned *cis*-elements in the promoters of the IDED genes is in accordance with a possible regulation by FIT and ethylene, although their functionality has to be demonstrated by further analyses.

Induction of IDED genes, ferric reductase activity, and subapical root hair development in the ethylene mutant ein2-1

In order to look further into the role of ethylene in the regulation of physiological (gene expression and ferric reductase activity) and morphological (root hair development) responses to Fe deficiency, some experiments were carried out with the ethylene-insensitive mutant *ein2-1*. In the *ein2-1* mutant, ethylene induced the expression of relevant Fe-related IDED genes (Fig. 5) and enhanced the ferric reductase activity, but did not induce the formation of subapical root hairs (Fig. 4). In this mutant, Fe deficiency had a similar effect to ethylene since it also induced the expression of both Fe acquisition genes (Fig. 5) and enhanced ferric reductase activity (Fig. 4; Schmidt *et al.*, 2000*b*), but not the formation of subapical root hairs (not shown; Schmidt and Schikora, 2001).

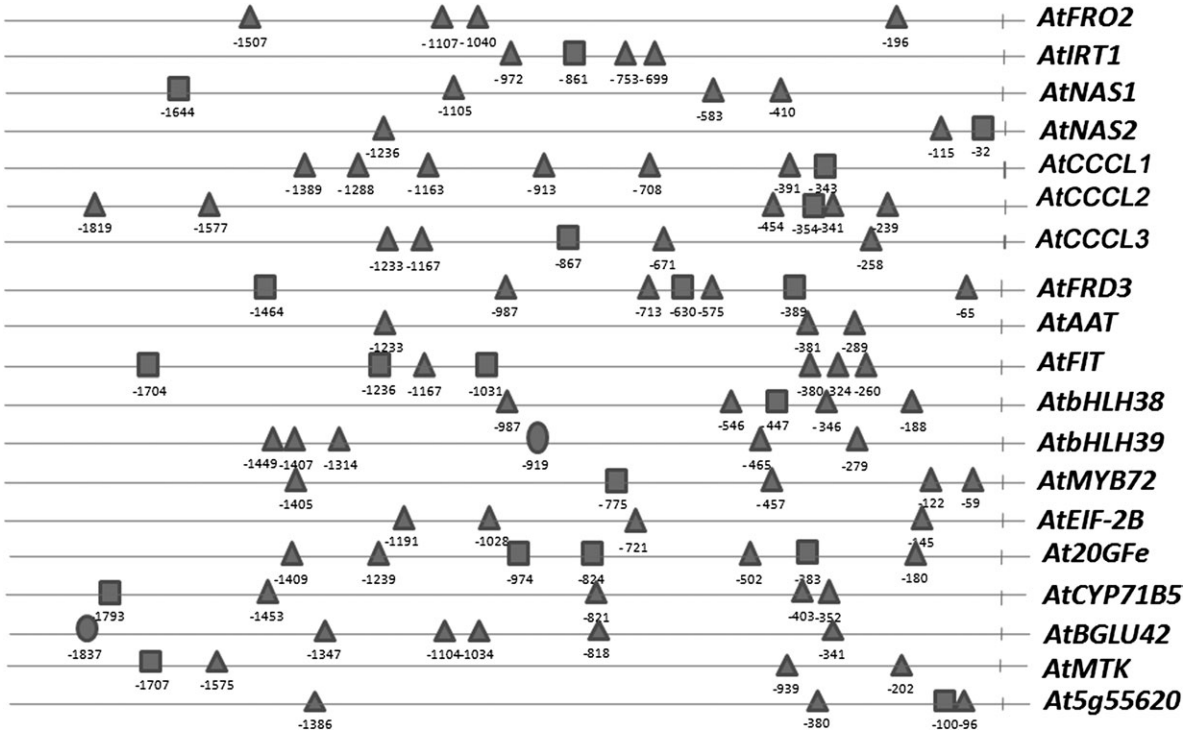

Fig. 3. Occurrence of ethylene- and bHLH transcription factor-responsive elements in the promoter regions of IDED genes. A stretch of 2000 bp upstream of the transcription initiation site was examined. The ethylene responsive elements AWTTCAAA and GCCGCC are represented by square and oval symbols, respectively. The bHLH recognition sequence CANNTG is indicated by triangles. The numbering below each symbol indicates the position of each motif above the site of initiation of transcription.

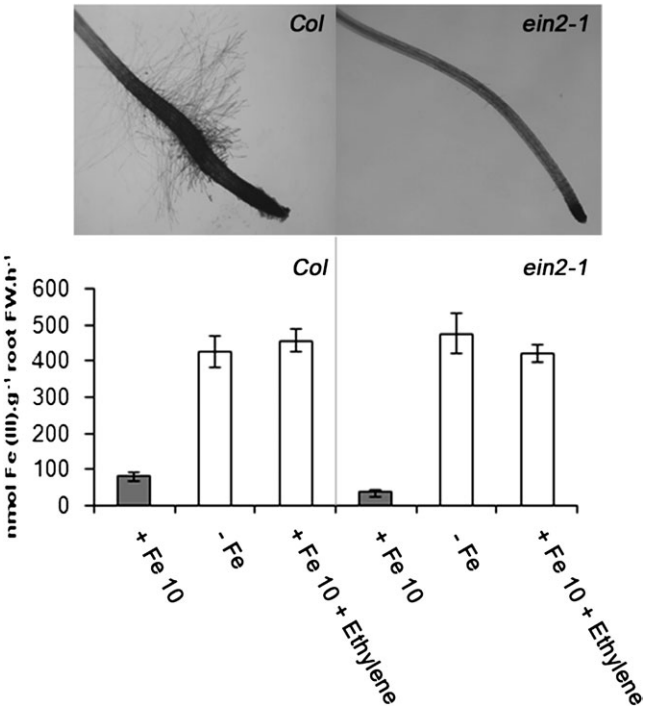

Fig. 4. Effect of ethylene on the development of subapical root hairs (top) and of ethylene and Fe deficiency on ferric reductase activity in the wild type 'Columbia' and in the insensitive mutant *ein2-1* (bottom). Pictures show roots of ethylene-treated plants. Plants were grown in nutrient solution with 10 μM Fe (+Fe10) and some of them were transferred for 24 h to nutrient solution without Fe (–Fe) or with ethylene (+Fe10+ethylene).

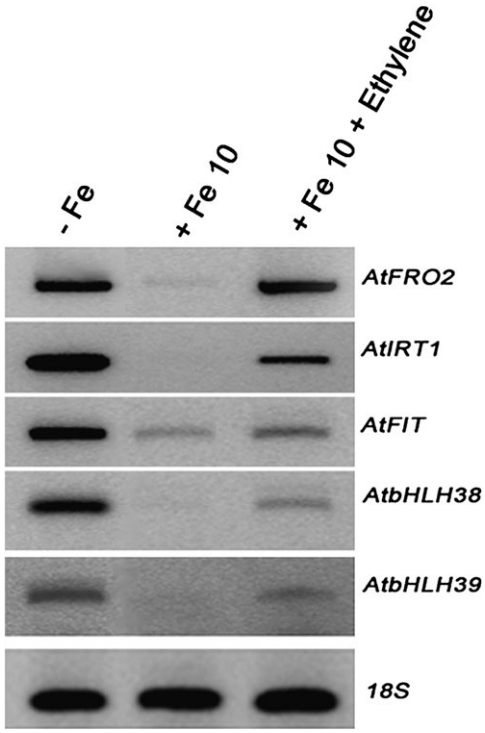

Fig. 5. Effect of Fe deficiency and ethylene treatment on the expression of several Fe acquisition genes in the ethylene-insensitive mutant *ein2-1*. Some plants grown in nutrient solution with 10 μM Fe (+Fe10) were transferred for 24 h to nutrient solution without Fe (–Fe) or with ethylene (+Fe10+ethylene). RT-PCR was performed using total RNA from roots as template and gene-specific primers to amplify partial cDNAs from the genes indicated in the figure. 18S cDNA was amplified as an internal control.

Induction of IDED genes by NO

Graziano and Lamattina (2007) showed that the Fe acquisition genes *SlFER* (tomato *FIT* homologue), *SlFRO1*, and *SlIRT1* were up-regulated by GSNO (NO precursor), similarly to what occurred with the ethylene precursor ACC (Lucena *et al.*, 2006). Based on this, tests were perfromed to determine whether the Fe-related IDED genes identified in the present work are also up-regulated by NO. For this, *Arabidopsis* 'Columbia' plants grown with low levels of Fe were treated for 24 h with GSNO. As shown in Fig. 6, all the Fe-related IDED genes were also up-regulated by GSNO, similarly to what occurred with ethylene (Fig. 2b).

Induction of ethylene synthesis genes in response to Fe deficiency

The occurrence of IDED genes, such as those identified in this work, suggests an enhanced ethylene production for their full expression and it is already known that ethylene synthesis increases in Fe-deficient roots of Strategy I plants (Romera *et al.*, 1999; Waters and Blevins, 2000; Li and Li, 2004; Molassiotis *et al.*, 2005; Zuchi *et al.*, 2009). A possibility is that Fe deficiency up-regulates genes for key enzymes of the ethylene biosynthetic pathway. To test this hypothesis, the abundance of transcripts from genes coding for SAM synthetase, ACC synthase, and ACC oxidase was determined in roots of plants grown under Fe-sufficient and Fe-deficient conditions. Two genes for SAM synthetase, *AtSAM1* and *AtSAM2*; four genes for ACC synthase, *AtACS4*, *AtACS6*, *AtACS9*, and *AtACS11*; and three for ACC oxidase, *AtACO1*, *AtACO2*, and *AtACO3*, were examined. As shown in Fig. 7, all ethylene synthesis genes, except *AtACS11* and *AtACO3*, responded with increased transcript abundance after 1 d of Fe deprivation. Additionally, the *AtMTK* gene encoding 5-methylthioribose kinase, an enzyme involved in methionine recycling for sustained ethylene production (Sauter *et al.*, 2004; Bürstenbinder *et al.*, 2007), was also induced by Fe deficiency, as detected by the microarray analysis (Table 2) and confirmed by RT-PCR (Fig. 2a).

Induction of ethylene signalling genes in response to Fe deficiency

Besides the increased ethylene production by Fe-deficient roots, the possibility exists that Fe deficiency could also alter the ethylene perception and transduction machinery. To test this hypothesis, the expression of several ethylene signalling genes (*AtETR1*, *AtCTR1*, *AtEIN2*, *AtEIN3*, *AtEIL1*, and *AtEIL3*) was determined in roots of plants grown under

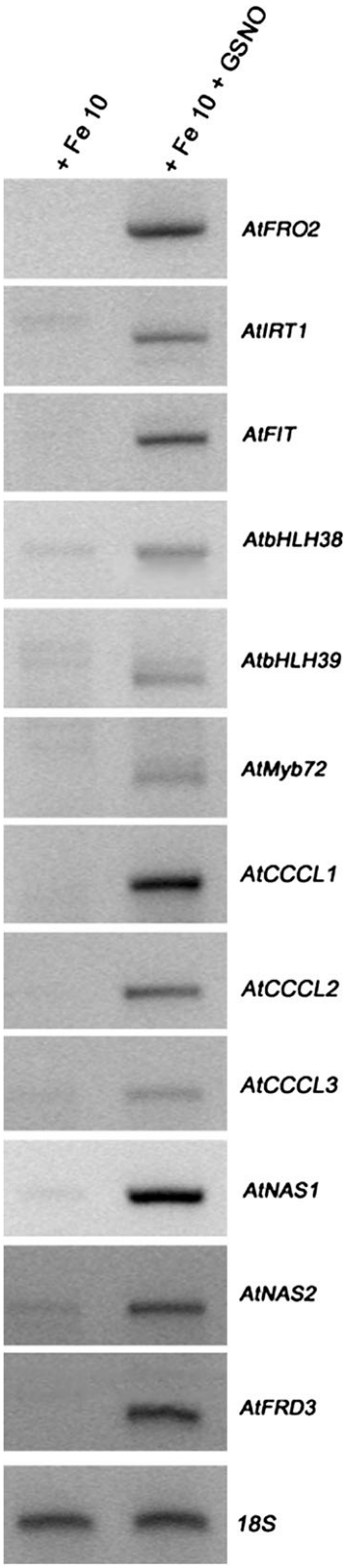

Fig. 6. Effect of GSNO treatment on the expression of Fe-related genes. Plants were grown in nutrient solution with 10 μM Fe (+Fe10) and half of them were treated during the last 24 h with GSNO 100 μM (+Fe10+GSNO). RT-PCR was performed using total RNA from roots as template and gene-specific primers to amplify partial cDNAs from the genes indicated in the figure. 18S cDNA was amplified as an internal control.

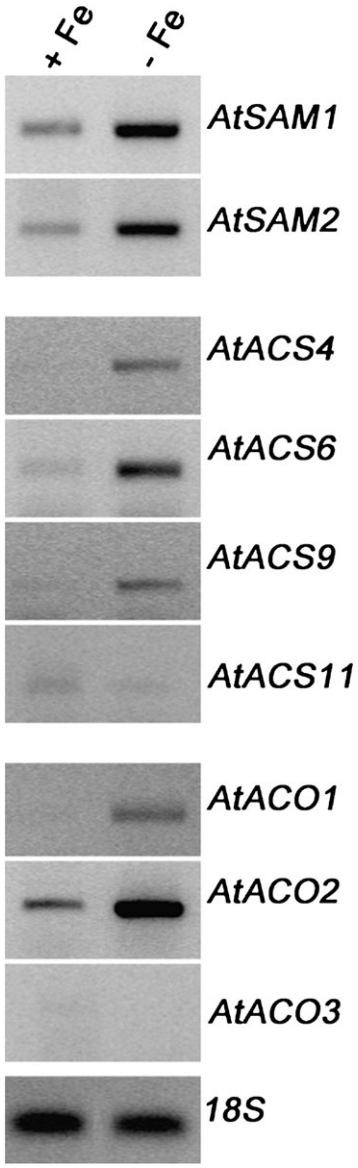

Fig. 7. Effect of Fe deficiency on the expression of several ethylene synthesis genes. Plants grown in nutrient solution with 10 μM Fe were transferred for 24 h to nutrient solution with 40 μM Fe (+Fe40) or without Fe (–Fe). RT-PCR was performed using total RNA from roots as template and gene-specific primers to amplify partial cDNAs from the genes indicated in the figure. 18S cDNA was amplified as an internal control.

Fe-sufficient and Fe-deficient conditions. As shown in Fig. 8, all ethylene signalling genes tested responded with increased transcript abundance after 1 d of Fe deprivation.

Discussion

In a previous work (Lucena *et al.*, 2006), ethylene was shown to be involved in the up-regulation, under Fe-deficient conditions, of three relevant genes for Fe acquisition in *Arabidopsis*: the transcription factor *AtFIT*, the ferric reductase *AtFRO2*, and the iron transporter *AtIRT1*. The results presented in this work extend the role of

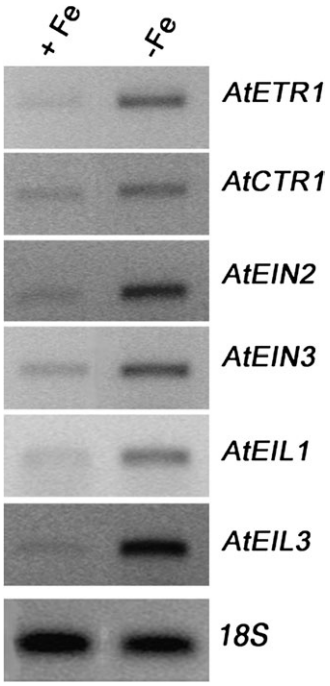

Fig. 8. Effect of Fe deficiency on the expression of several genes involved in the ethylene transduction pathway. Plants grown in nutrient solution with 10 μM Fe were transferred for 24 h to nutrient solution with 40 μM Fe (+Fe40) or without Fe (–Fe). RT-PCR was performed using total RNA from roots as template and gene-specific primers to amplify partial cDNAs from the genes indicated in the figure. 18S cDNA was amplified as an internal control.

ethylene in the regulation of Fe deficiency-induced genes to 16 new genes. Most of these IDED genes were selected by microarray analysis (Table 2) and their ethylene dependence was confirmed in further experiments with ethylene inhibitors and ethylene treatment (Fig. 2). Some of them, such as *AtFRO2*, *AtbHLH38*, and *AtbHLH39*, were not identified by the microarray analysis (*AtFRO2* and *AtbHLH38* are not included in the ATH1 chip) but by RT-PCR (Fig. 2; Lucena *et al.*, 2006; Romera, 2008). The total number of IDED genes identified so far is 19, which is small compared with the 116 genes up-regulated by Fe deficiency (Supplementary Table S1 at *JXB* online). However, most of the IDED genes identified in this work have very significant roles in Fe acquisition and homeostasis, as discussed later. Nevertheless, since the criterion used to identify IDED genes was rather restrictive—only genes that were negatively affected by two different ethylene inhibitors were selected—their number is expected to increase in future studies.

Among the IDED genes identified are the bHLH transcription factors *AtFIT* (*AtbHLH29*), *AtbHLH38*, and *AtbHLH39*. These bHLH transcription factors are considered as the activators of the main genes involved in Fe acquisition, such as *AtFRO2* and *AtIRT1* (Colangelo and Guerinot, 2004; Jakoby *et al.*, 2004; Bauer *et al.*, 2007; Walker and Connolly, 2008; Yuan *et al.*, 2008). Yuan *et al.* (2008) have shown that the transcription of *AtFRO2* and *AtIRT1* is directly activated by a complex of FIT/bHLH38 or FIT/bHLH39. In this work, it has been found that the up-regulated expression of *AtFIT*, *AtbHLH38*, and *AtbHLH39* in Fe-deficient plants is diminished by ethylene inhibitors (Fig. 2a). On the other hand, ethylene up-regulated their expression in plants grown in low Fe conditions (Fig. 2b), which confirms previous results obtained upon ACC treatment (Lucena *et al.*, 2006; Romera, 2008).

The regulation of the above bHLH transcription factors by ethylene would imply that those IDED genes dependent on FIT (and on bHLH38 or bHLH39) could consequently be affected by ethylene treatments. Besides *AtFRO2* and *AtIRT1*, other IDED genes have been reported to depend mainly on FIT, such as *AtNAS1*, *AtAAT*, *AteIF-2B*, *At2OGFe*, *AtCYP71B5*, *AtBGLU42*, and *AtMTK* (Colangelo and Guerinot, 2004). All these genes have in their promoters bHLH transcription factor-responsive elements (Fig. 3) that could mediate regulation by FIT and also by bHLH38 or bHLH39. Some of them (*AtNAS1*, *At2OGFe*, *AtCYP71B5*, *AtBGLU42*, and *AtMTK*) also possess EREs (Fig. 3) that could support regulation by ethylene itself. *AtNAS1* encodes an NAS gene, involved in the synthesis of nicotianamine, a chelator of metals that plays a role in the internal transport of Fe and other metals (Suzuki *et al.*, 1999, 2001; Weber *et al.*, 2004; Klatte *et al.*, 2009). *AtMTK* encodes a 5-methylthioribose kinase, an enzyme of the 'Yang cycle' that allows methionine recycling for ethylene synthesis and is essential for sustained ethylene production (Sauter *et al.*, 2004; Bürstenbinder *et al.*, 2007). Therefore, it could be necessary for the enhanced production of ethylene described in Fe-deficient roots of Strategy I plants (Romera *et al.*, 1999; Waters and Blevins, 2000; Li and Li, 2004; Molassiotis *et al.*, 2005; Zuchi *et al.*, 2009). *AtCYP71B5* encodes a protein of the cytochrome P450 monooxygenase family. This and other cytochrome P450-like proteins have been reported to be induced by Fe deficiency (Colangelo and Guerinot, 2004; Buckhout *et al.*, 2009). Although their precise function in Fe homeostasis is unknown, Van de Mortel *et al.* (2008) suggested a possible role in the reinforced lignification of the vascular cylinder to prevent excess efflux of metals. The other IDED genes depending on FIT (Colangelo and Guerinot, 2004) encode an amino acid transporter (*AtAAT*), a putative eukaryotic translation initiation factor (*AteIF-2B*), a protein of the 2OG-Fe(II) oxygenase family (*At2OGFe*), and a β-glucosidase (*AtBGLU42*) that, at the moment, have not been related to Fe homeostasis.

The transcription factor *AtMYB72*, another IDED gene, has been described as partially dependent on FIT (Colangelo and Guerinot, 2004), which could explain its dependence on ethylene (Table 2, Fig. 2). Nonetheless, this gene also has an ERE in its promoter (Fig. 3). *Arabidopsis myb72* mutants show more sensitivity to Fe-deficient conditions than wild-type plants, which suggests that this transcription factor is necessary for the development of a correct response to Fe deficiency (Van de Mortel *et al.*, 2008). On the other hand, MYB72 is involved in induced systemic resistance, where it has been shown to interact physically with the ETHYLENE INSENSITIVE 3-like protein EIL3 (Van der Ent *et al.*, 2008), a protein related to ethylene signalling that is also induced (at least, at the

transcriptional level) under Fe deficiency (Fig. 8). Since ethylene is involved in the regulation of Fe deficiency-induced genes, this raises the question of whether the tandem MYB72–EIL3 also plays a regulatory role in Fe deficiency responses.

In addition to the above-mentioned genes, whose dependence on ethylene could be mainly, or partly, related to their regulation by FIT, several IDED genes were found that, as far as is known, have not been related to FIT (Colangelo and Guerinot, 2004). These genes are *AtNAS2*, *AtCCCL1*, *AtCCCL2*, *AtCCCL3*, and *AtFRD3*; all of them contain EREs in their promoters that could explain a regulation by ethylene itself (Fig. 3). These genes are all involved in different aspects of Fe transport. *AtNAS2*, as well as *AtNAS1*, encodes an NAS that catalyses the synthesis of nicotianamine, a chelator for the internal transport of Fe (Suzuki *et al.*, 1999, 2001; Weber *et al.*, 2004; Klatte *et al.*, 2009). *AtCCCL1*, *AtCCCL2*, and *AtCCCL3*, that show sequence similarity to nodulins, encode putative membrane proteins similar to the yeast CCC1, which is involved in the transport of Fe^{2+} from the cytosol to the vacuole for storage (Li *et al.*, 2001). Curiously, some of these genes are down-regulated during the first hours of Fe deficiency in other microarrays (Dinneny *et al.*, 2008; Buckhout *et al.*, 2009; Yang *et al.*, 2010). A possible explanation for this is that, at first, these genes are down-regulated to keep the scarce Fe of Fe-deficient cells in the cytosol. Later on, they would be up-regulated to accumulate in vacuoles the probable Fe excess originating from the induction of Fe acquisition genes. The time course of the expression of these *CCCL* genes was analysed at 6, 12, and 24 h after Fe deficiency and it was found that they are expressed at very low levels at 6 h and 12 h after Fe deficiency, then being up-regulated at 24 h after Fe deficiency, which agrees with the above suggestion (data not shown). *AtFRD3* is another gene whose full induction by Fe deficiency seems to require ethylene (Table 2, Fig. 2). *AtFRD3* encodes a protein responsible for the loading into the xylem of citrate, an Fe^{3+} chelator, which is essential for the correct distribution of Fe throughout plant tissues (Durrett *et al.*, 2007).

In summary, the Fe deficiency-induced genes identified as ethylene dependent in this study include the main regulators of the Fe deficiency responses (*FIT*, *bHLH38*, and *bHLH39*); the ferric reductase and the Fe transporter on the plasma membrane (*FRO2* and *IRT1*); Fe transporters on the tonoplast (*CCCL*); internal Fe transport (*NAS*); translocation of Fe in the xylem (*FRD3*); as well as genes involved in ethylene synthesis (*MTK*). In addition to its influence on the Fe-related genes mentioned above, ethylene is involved in the formation of subapical root hairs (Fig. 4), one of the responses to Fe deficiency (Romera and Alcántara, 2004). Taken together, all these results suggest that ethylene acts as a coordinator of a significant part of the responses to Fe deficiency at the whole-plant level.

To gain insight into the mechanism by which ethylene regulates Fe deficiency genes, the induction of selected IDED genes and the development of subapical root hairs were examined in the *Arabidopsis* ethylene-insensitive mutant *ein2-1*. The *ein2-1* mutation did not impair the up-regulation of the IDED genes by either Fe deficiency or ethylene treatment (Fig. 5), though it abolished the development of subapical root hairs under both treatments (Fig. 4; Schmidt and Schikora, 2001; Romera and Alcántara, 2004). At first sight, these results could suggest that ethylene is involved in the development of subapical root hairs but not in the regulation of the expression of Fe acquisition genes, as proposed by Schmidt *et al.* (2000*b*). This interpretation would be correct if it is assumed that *ein2* mutations block absolutely all the responses to ethylene, but this is not the case: when *ein2-1* plants grown under low Fe conditions were treated with ethylene, Fe acquisition genes were up-regulated (Fig. 5), showing that ethylene can induce them even in the absence of a functional EIN2 protein. These results are in accordance with an ethylene signalling network far more complex than previously thought, in which an alternative route to EIN2 could exist (Cho and Yoo, 2009). The recent works by Yoo *et al.* (2008) and Li *et al.* (2009) clearly show that ethylene signals can be transduced to transcriptional events via both *ein2*-dependent and -independent pathways. Furthermore, it was recently found that Fe deficiency induced the expression of ethylene transduction genes (*EIN3*, *EIL1*, and *ERF1*) acting downstream of *EIN2* in the *ein2-1* mutant (data not shown). These results also show that ethylene signalling is not totally impaired in the *ein2* mutant.

In addition to ethylene, Graziano and Lamattina (2007) have shown that NO can also up-regulate important Fe acquisition genes in tomato, such as *SlFER*, *SlFRO1*, and *SlIRT1*. Based on this, tests were performed to determine whether NO also up-regulates the Fe-related IDED genes found in this work. For this, GSNO (an NO precursor) was applied to Fe-sufficient plants and the results obtained showed that all the Fe-related IDED genes studied were also responsive to NO (Fig. 6). Besides ethylene and NO, salicylic acid (SA) has also been shown to be involved in the up-regulation of *AtbHLH38* and *AtbHLH39* (Kang *et al.*, 2003) and in the enhanced ferric reductase activity of Fe-deficient peach rootstocks (Molassiotis *et al.*, 2005). Similarly, auxin has also been shown to be involved in the regulation of some Fe deficiency responses (Romera and Alcántara, 2004). Since auxin (Romera and Alcántara, 2004), NO (Wang *et al.*, 2009), and SA (Molassiotis *et al.*, 2005) can increase ethylene production, the possibility exists that all of them could act through ethylene, at least in some of the responses, but this deserves further research.

A second objective of this work was to analyse whether Fe deficiency up-regulates the expression of genes involved in ethylene biosynthesis and signalling. As shown in Figs 7 and 8, Fe deficiency up-regulates the expression of genes involved in ethylene synthesis (SAM synthetase genes, *AtSAM1* and *AtSAM2*; ACC synthase genes, *AtACS4*, *AtACS6*, and *AtACS9*; and ACC oxidase genes, *AtACO1* and *AtACO2*) and signalling (*AtETR1*, *AtCTR1*, *AtEIN2*, *AtEIN3*, *AtEIL1*, and *AtEIL3*) in the roots. The higher

expression of ethylene synthesis genes would explain the higher ethylene production of Fe-deficient roots (Romera *et al.*, 1999; Waters and Blevins, 2000; Li and Li, 2004; Molassiotis *et al.*, 2005; Zuchi *et al.*, 2009). It should be noted that, besides SAM synthetase, ACC synthase, and ACC oxidase genes (Fig. 7), other genes related to ethylene synthesis are also induced under Fe deficiency conditions, such as the *AtMTK* gene (Fig. 2). Buckhout and Thimm (2003) also found enhanced expression of ACC oxidase and SAM synthetase genes under Fe deficiency. Similarly, Li *et al.* (2008) found enhanced expression of proteins related to methionine and SAM synthesis under Fe deficiency in tomato plants. All these results indicate that the machinery for ethylene synthesis is changed under Fe deficiency. Ethylene production also increases under other nutrient deficiencies, such as phosphorus deficiency and potassium deficiency (Lynch and Brown, 1997; Gilbert *et al.*, 2000; Shin and Schachtman, 2004; Jung *et al.*, 2009). Shin and Schachtman (2004) have found enhanced expression of ACC oxidase genes, such as *AtACO1*, under potassium starvation. In the same way, Hernández *et al.* (2007) have found enhanced expression of an ACC oxidase gene in P-deficient common bean roots.

Besides changing ethylene production, nutrient deficiencies can also change ethylene sensitivity. There are several works in the literature showing that some nutrient deficiencies can enhance the sensitivity to ethylene. He *et al.* (1992) showed enhanced sensitivity to ethylene of nitrogen- or phosphorus-deficient *Zea mays* plants. Similarly, other authors have found enhanced sensitivity to ethylene of phosphorus-deficient *Phaseolus vulgaris* (Borch *et al.*, 1999) or *A. thaliana* (Ma *et al.*, 2003) plants. In this work, the higher expression of several ethylene signalling genes under Fe deficiency (Fig. 8) suggests that the sensitivity to ethylene can change in Fe-deficient roots as part of the mechanism of ethylene regulation of the Fe deficiency responses. The results are in accordance with Ciardi and Lee (2001), who suggest that 'increases in receptors at times of increased ethylene synthesis seem to be a general phenomenon' since 'some receptors are known to be ethylene-inducible'.

In conclusion, in this work complementary aspects of the relationship between ethylene and NO on Fe acquisition and homeostasis have been studied, including the identification of new Fe deficiency-induced ethylene (and NO)-dependent genes, the expression of Fe acquisition genes in an ethylene-insensitive mutant, as well as the effect of Fe deficiency on ethylene synthesis and signalling genes. The results obtained reveal an extended influence of ethylene and NO on Fe acquisition and homeostasis in Strategy I plants, and a wide influence of Fe deficiency on ethylene synthesis and signalling.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Genes up-regulated by iron deficiency.

Acknowledgements

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