β -Aminobutyric Acid (BABA)-Induced Resistance in *Arabidopsis thaliana*: Link with Iron Homeostasis

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Submitted 19 May 2014. Accepted 10 July 2014.

β -Aminobutyric acid (BABA) is a nonprotein amino acid inducing resistance in many different plant species against a wide range of abiotic and biotic stresses. Nevertheless, how BABA primes plant natural defense reactions remains poorly understood. Based on its structure, we hypothesized and confirmed that BABA is able to chelate iron (Fe) in vitro. In vivo, we showed that it led to a transient Fe deficiency response in *Arabidopsis thaliana* plants exemplified by a reduction of ferritin accumulation and disturbances in the expression of genes related to Fe homeostasis. This response was not correlated to changes in Fe concentrations, suggesting that BABA affects the availability or the distribution of Fe rather than its assimilation. The phenotype of BABA-treated plants was similar to those of plants cultivated in Fe-deficient conditions. A metabolomic analysis indicated that both BABA and Fe deficiency induced the accumulation of common metabolites, including *p*-coumaroylagmatine, a metabolite previously shown to be synthesized in several plant species facing pathogen attack. Finally, we showed that the protective effect induced by BABA against *Botrytis cinerea* was mimicked by Fe deficiency. In conclusion, the Fe deficiency response caused by BABA could bring the plant to a defense-ready state, participating in the plant resistance against the pathogens.

Iron (Fe), an essential nutrient for both animals and plants, is the fourth most abundant element in the Earth's crust. Paradoxically, it is the third most limiting nutrient for plant development, mainly due to its poor solubility under aerobic conditions. To deal with the limiting amounts of bioavailable Fe, plants have evolved two different strategies to assimilate the metal from the soil. Non-grass plants such as *Arabidopsis thaliana* use the strategy I mechanism, which includes three steps: i) proton release in order to solubilize Fe³⁺ in the soil, ii) re-

duction of Fe³⁺ by a membrane-bound Fe³⁺ chelate reductase (mainly ferric reductase [FRO]2 in *A. thaliana*) (Robinson et al. 1999), and iii) subsequent transport of the resulting Fe²⁺ into the plant root cells by an Fe²⁺ transporter. In *A. thaliana*, the major root Fe transporter is IRT1 (Vert et al. 2002). Strategy II is exclusively used by grasses and involves direct Fe³⁺ chelation by phytosiderophores (PS) and the subsequent uptake of Fe³⁺-PS complexes via specific transporters. Once assimilated by roots, Fe is utilized, sequestered, or transported to other organs. Because Fe³⁺ ions are often precipitated in ferric hydroxides and Fe²⁺ free ions can catalyze the synthesis of hydroxyl radicals through the Fenton reaction, Fe is associated with various molecules such as organic acids. In the xylem sap, Fe is mostly associated with citrate whereas, in the phloem, it might be chelated to nicotianamine (NA) (von Wiren et al. 1999), a nonproteogenic amino acid. More generally, NA plays a key role in iron homeostasis. Indeed, in strategy II plants, NA is an intermediate for the synthesis of mugineic acid family PS. In strategy I plants, NA contributes to iron long-distance circulation in phloem and transfer within the cells, and may act as a sensor for iron availability (Curie et al. 2009; Koen et al. 2013; Schuler et al. 2012). At the subcellular level, plants can cope with Fe scarcity and toxicity by buffering and storing Fe in the apoplast, vacuoles, and plastids. Ferritins are key plastidial proteins able to sequester between 2,000 and 4,000 Fe³⁺ atoms by storing them in their central cavity. These proteins are essential to protect plants against oxidative stresses and to provide iron during developmental processes or environmental stresses (Briat et al. 2010).

Under Fe limitation, plants initiate physiological and morphological responses to increase Fe mobilization and uptake from the soil. In *A. thaliana*, expression of *IRT1*, *FRO2*, and the root specific *bHLH* FER-like iron-deficiency-induced transcription factor (*FIT*) is modulated upon Fe-deficiency. The expression of *FRO2* and *IRT1* is regulated by *FIT* at the transcriptional and post-transcriptional level, respectively (Colangelo and Guerinot 2004). Moreover, *FRO2* is regulated post-transcriptionally by an unknown mechanism (Connolly et al. 2002) and both *IRT1* and *FRO2* expression are induced by local Fe (Vert et al. 2003). Recently, it was shown that *IRT1* is regulated by a monoubiquitin- and clathrin-dependent trafficking in root epidermal cells, enabling its low expression at the cell surface to cope with the toxicity induced by other metal sub-

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*The e-Xtra logo stands for “electronic extra” and indicates that five supplementary figures and a supplementary table are published online.

strates of IRT1 as cadmium (Barberon et al. 2011). Fe deficiency also triggers a decrease in the ferritin level (Petit et al. 2001) and an increase of *NA SYNTHASE* (*NAS*) transcript accumulation in the roots or in the shoots, depending on the isoform (Bauer et al. 2004). In particular, among the four *NAS* genes present in the *A. thaliana* genome, recent data support a key role of AtNAS4 in iron distribution and proper response to iron deficiency (Koen et al. 2013; Palmer et al. 2013). More generally, between the four AtNASs, the plant response to iron deficiency appears to be mostly determined by AtNAS4 (Koen et al. 2013; Schuler and Bauer 2011).

Plants interact with a variety of microorganisms that are potentially pathogenic. During the interaction, certain microbes produce siderophores which are normally secreted to assimilate Fe from their environment. Siderophores can exert beneficial or negative effects on the host plant. First, because siderophores are able to significantly increase Fe solubility in the soil, they can have a positive effect on plant growth by facilitating Fe assimilation (Chen et al. 1998). For example, pyoverdins, siderophores secreted by fluorescent pseudomonads can be directly imported into *A. thaliana* roots and, subsequently, promote an increase of Fe concentration in planta (Vansuyt et al. 2007). Second, siderophores are also required for the pathogenicity of bacteria and fungi (Expert 1999; Haas et al. 2008). Indeed, by releasing siderophores in plant tissues, microorgan-

isms can import the plant Fe. This process is favored by the highest affinity of microbial siderophores for Fe compared with those of phytosiderophores and other plant Fe carriers. Recent studies showed that plants can counteract this process by establishing an Fe retention strategy characterized by a rise in ferritin synthesis and Fe acquisition (Dellagi et al. 2009; Segond et al. 2009). Notably, it was shown that the induction of the expression of the *FERRITIN1* (*FER1*) gene is required for the basal resistance of *A. thaliana* against *Dickeya dadantii*, a bacterium which causes soft rot on a large range of host plant species. In addition, *FRO2* or *IRT1* transcript accumulation is also induced in response to *D. dadantii* infection and bacterial siderophores such as deferrioxamine (Aznar et al. 2014). Recently, the existence of a link between plant iron status and susceptibility or resistance to diseases was highlighted by Kieu and associates (2012). They demonstrated that *A. thaliana* plants grown in Fe-deficient conditions were more resistant to *D. dadantii* and *Botrytis cinerea* infections. The exact mechanism of Fe-starvation-induced resistance in response to *D. dadantii* is not yet elucidated. It has been proposed that this phenomenon might be related to the decrease in plant Fe availability rather than an amplification of the plant defense responses.

β -Aminobutyric acid (BABA) is a nonproteogenic amino acid that potentiates plant defense responses via priming mechanisms and confers resistance to a variety of biotic and abiotic

Fig. 1. Analysis of the Fe³⁺-chelating properties of BABA by **A**, electrochemistry using turning-type electrode and **B**, UV/visible spectroscopy. **C**, Comparison of the results obtained by the two techniques and **D**, BABA-Fe³⁺ complex model. In this model, generated with ChemBioDraw Ultra 12.0, three molecules of BABA (in black) chelate one atom of Fe³⁺ (in blue) through their carboxylic and amine functions (the six bonds are represented by the red lines). Results presented in A, B, and C are from one of three representative experiments.

stresses (Cohen 1994; Cohen et al. 1999; Jakab et al. 2005; Ton et al. 2005; Zimmerli et al. 2000, 2001). Application of BABA to *A. thaliana* plants induces resistance against both biotrophic and necrotrophic pathogens. Nevertheless, this broad spectrum of effectiveness is based on different priming mechanisms. Against some pathogens, such as the bacterial leaf pathogen *Pseudomonas syringae*, BABA-induced resistance is based on the priming of defense reactions that are regulated by the plant hormone salicylic acid (SA) and the defense regulatory protein NPR1. During these interactions, the priming manifests as a faster and stronger induction of SA-inducible defense genes, such as *PR1*. However, against some fungi and oomycetes, BABA-induced resistance operates independently of SA and NPR1. In these cases, the induced protection is based on an abscisic acid (ABA)-dependent priming of cell-wall-related defense such as the formation of papillae that blocks pathogen invasion (Conrath et al. 2006; Ton and Mauch-Mani 2004; Ton et al. 2005).

In addition to numerous studies revealing that BABA is able to modulate the expression of defense-related genes and to potentiate plant defense reactions, several teams have recently attempted to explore BABA mode of action. It was suggested that BABA induces a stress-induced morphogenic response in *A. thaliana* that could be responsible for the BABA-induced resistance (Wu et al. 2010). The same group showed that BABA-induced chromatin modification is correlated with BABA priming of *A. thaliana* pattern-triggered immunity, suggesting that epigenetics could be important for BABA priming (Po-Wen et al. 2013). In addition, it was shown that BABA treatment leads to a strong accumulation of pipelicolic acid, an endogenous modulator of priming that likely plays a role in BABA-induced resistance against bacteria (Návarová et al. 2012). More recently, Luna and associates (2014) brought evidence that IBI1, an aspartyl-tRNA synthetase (AspRS), is a plant receptor of BABA. Binding of BABA to the L-Asp-binding site of IBI1 leads to a switch from its canonical AspRS activity to its noncanonical defense activity. They also suggest that the inhibitory effect of BABA on plant growth may be due to the accumulation of uncharged tRNA^{Asp}, which triggers phosphorylation of the translation initiation factor eIF2 α .

In the present study, we provide new elements that could explain BABA mode of action by demonstrating that BABA displays *in vitro* Fe-chelating properties. To investigate whether this property might account for BABA effects in planta, we analyzed the putative incidence of BABA on plant Fe homeostasis. We found that the pretreatment of *A. thaliana* plantlets with BABA triggered drastic changes in the expression of Fe-related genes and proteins, as well as a macroscopic phenotype resembling those induced by a transient or drastic Fe deficiency. Furthermore, BABA triggered the accumulation of several metabolites also accumulating in plants exposed to Fe deficiency. Measurement of Fe level in BABA-treated plants suggested that BABA acts by modifying in planta Fe availability rather than Fe assimilation. Finally, we provide evidence that the Fe status is an important determinant for plant resistance against *B. cinerea*. Collectively, our study highlights a new mode of action of BABA and further supports the recent concept (Aznar et al. 2014) that iron scavenging is a mechanism of immunity activation in plants.

RESULTS

BABA chelates Fe³⁺ ions by forming a stable hexacoordinated complex.

Based on its structure, BABA is a potential Fe chelator, as described for other amino acids (Mandado and Cordeiro 2010;

Pizarro et al. 2002). Therefore, the possibility that BABA forms a complex with Fe³⁺ was first monitored by linear voltammetry using a turning-type electrode (Fig. 1A). The voltammogram of a 5×10^{-3} M Fe³⁺ solution enriched with increasing concentrations of BABA shows that, in the absence of BABA (black curve), a typical voltammetric wave was observed from 0.35 to 0.7 V. The height of this wave was drastically reduced following the addition of one equivalent of BABA (blue curve) and disappeared from three equivalents of the amino acid (red curve). Simultaneously, a new wave at less positive potentials corresponding to the reduction of the BABA-Fe³⁺ complexes was observed with increasing concentrations of BABA. Hence, the complex formation between BABA and iron facilitates the Fe³⁺ reduction. Using these voltammetric measurements and according to the method proposed by Stephan and associates (1996), we calculated an Fe(III)-BABA stability constant of 30.07, which is more than the Fe(III)-citric acid and the Fe(III)-EDTA stability constants (11.85 and 25.7, respectively) (Furia 1972).

To confirm these results, the formation of BABA-Fe³⁺ complexes was analyzed by UV-visible spectroscopy (Fig. 1B). The UV-visible spectrum of Fe³⁺ displayed four bands at wavelengths below 400 nm (black curve). When one (blue curve), two (green curve), three (red curve), or four (purple curve) equivalents of BABA were added in the medium, two additional bands at 380 and 479 nm were observed. Appearance of these bands highlights the formation of a complex between BABA and Fe³⁺.

The data obtained by linear voltammetry (blue curve for Fe³⁺ alone and black curve for BABA-Fe³⁺ complexes) and spectroscopy at 479 nm (orange curve) are combined in Figure 1C. This wavelength was chosen because Fe³⁺ alone does not absorb such a value. The curves clearly show that both methods gave similar data: addition of BABA results in the formation of a complex between the amino acid and Fe³⁺. The curves did not further evolve from the addition of three equivalents of BABA, indicating that the complex involves three molecules of BABA for one Fe³⁺ ion. Based on this information, we generated a molecular model representing the putative structure of BABA and Fe³⁺ complexes (Fig. 1D). In this model, Fe³⁺ is hexacoordinated, each molecule of BABA binding the iron ion by their carboxylic and amino groups.

BABA modulates the accumulation of ferritins and *AtNAS4* transcripts in plants grown in soil.

The ability of BABA to chelate iron *in vitro* suggests that it might affect plant iron homeostasis. For this purpose, the accumulation of the iron storage protein ferritin was checked in plants grown in soil for 4 weeks and then soil drenched with water or 300 μ M BABA. Leaf tissues were collected 24 h after the soil drench. This procedure is classically used for BABA-induced resistance against pathogens (Jakab et al. 2005; Zimmerli et al. 2000). An immunoreactive band of 28 kDa corresponding to the monomeric form of ferritins was detected in both BABA-treated and control plants (Fig. 2A). However, ferritin accumulation was significantly reduced by 27% in BABA-treated plants (Fig. 2B). Because the major isoform among the four *A. thaliana* ferritins expressed in leaves is *AtFER1* (Ravet et al. 2009), the level of *FER1* transcript accumulation in BABA-treated plants was specifically monitored by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Compared with control plants, BABA-treated plants exhibited a significant reduction of *FER1* transcript accumulation (Fig. 2C). Therefore, BABA affects ferritin accumulation at both the transcript and protein levels.

To provide further arguments that BABA interferes with the regulation of components of iron homeostasis, its effect on the

accumulation of *NAS4* transcripts was also analyzed. As discussed above, the *NAS4* gene is upregulated in response to iron deficiency (Klatte et al. 2009; Koen et al. 2012) and the corresponding protein is required for proper response to iron deficiency (Koen et al. 2013). BABA treatment induced a 40% increase of *NAS4* transcript accumulation compared with control plants (Fig. 2D).

Macroscopic phenotypes of BABA-treated plants resemble those of plants facing iron deficiency.

The effects of BABA on ferritin and *AtNAS4* expression suggest that BABA affects plant iron homeostasis. To further investigate this hypothesis, we compared the responses triggered by BABA to those induced by iron deficiency. To allow an accurate control of the iron concentration in the growth medium, plants were cultivated in hydroponic conditions (Supplementary Fig. S1). Four treatments were performed: 4 week-old plants were treated for 24 h with 300 μ M BABA (BABA treatment) or the same volume of water (control) and were then transferred to a fresh nutritive solution without BABA for one additional week. In parallel, 4-week-old plants were exposed to

iron-deficiency conditions for 24 h and were then transferred either to a fresh nutrient solution without Fe (Fe 0 treatment) or to a fresh nutrient solution containing 50 μ M Fe (Fe 0 $\rightarrow$ Fe 50 treatment) for an additional week.

The macroscopic phenotype of the plant rosettes was observed after the 24-h pretreatment with BABA or Fe 0, and then 4 and 7 days after the plant transfer to the fresh medium. After 24 h of pretreatment, no difference was observed between control, BABA-, and Fe 0-treated plants (Fig. 3A). However, from 4 days after transfer, BABA-treated plants displayed a reduced growth compared with control plants and their leaf abaxial sides showed a red-purple pigmentation characteristic of anthocyanin production (Fig. 3B and C). Similar phenotypes were previously reported by Wu and associates (2010). Interestingly, the phenotype of the BABA-treated plants resembled that of Fe 0-treated plants. Indeed, from 4 days after transfer, Fe 0-treated plants were smaller and presented red-purple pigmentation of their leaf abaxial sides. The pigmentation was more pronounced than following BABA treatment. Moreover, they showed a chlorotic phenotype characteristic of iron deficiency (more pronounced at 7 days) (Fig. 3C). The plants trans-

Fig. 2. A and B, Ferritin accumulation and C, *FER1* and D, *NAS4* leaf transcript accumulation in plants grown in soil drenched with BABA. A, Immunodetection of ferritins by Western blotting was performed in protein extracts prepared from leaves of 4-week-old plants treated for 24 h with BABA or water. The Coomassie Blue (Coom.) and the Ponceau Red (Ponc.) stainings show equal loading and transfer, respectively. Results are from one of three representative experiments. B, Relative intensity (expressed in arbitrary units) of the immunoreactive bands from three independent Western blotting experiments were quantified using the Bio-Rad program Quantity one, version 4.6.7. Significant differences between the two treatments were determined by the Student's *t* test (* indicates $P < 0.05$). C and D, Measurement of transcripts accumulation by quantitative reverse-transcriptase polymerase chain reaction was performed from leaves of 4-week-old plants treated for 24 h with BABA or water. Data are expressed as mean normalized expression relative to the appropriate house-keeping gene *AtYLS8* (arbitrary unit) and are the means of triplicates ($\pm$ standard error) from one of three representative experiments. Significant differences between the two treatments were determined by the Student's *t* test (* indicates $P < 0.05$).

siently exposed to iron deficiency (Fe 0 → Fe 50 treatment) showed a slight delay in their growth 4 days after transfer. This delay was almost recovered 7 days after transfer, even though some leaves still exhibited anthocyanin accumulation. To complete this analysis, the plant shoots and roots were weighed and the leaf chlorophyll levels (Supplementary Fig. S2) were measured just after the 24-h pretreatment and 4 and 7 days after the transfer. From 4 days after the transfer, BABA-, Fe 0 → Fe 50-, and Fe 0-treated plants had reduced shoot and root weights

compared with control plants. At 7 days after the transfer, BABA-treated plants displayed shoot and root weights in between the plants cultivated in full or transient iron-deficiency conditions. Regarding chlorophyll content, a small decrease was measured for Fe 0 → Fe 50- and BABA-treated plants at 4 days after the transfer which was no longer observed after 7 days. The effects of the Fe 0 condition were more drastic, with a reduction of the chlorophyll content from 4 days after the transfer in agreement with the chlorotic phenotype observed in

Fig. 3. Macroscopic rosette phenotype of the plants treated with BABA or grown in iron-deficient conditions. After 4 weeks of hydroponic culture, plants were treated with 300 μ M BABA (BABA) or an equivalent volume of water (control) for 24 h and, then, were transferred to the nutritive solution without BABA for one supplementary week. For iron deficiency experiments, 4-week-old plants were treated for 24 h in the nutritive solution without Fe and then transferred either to the nutritive solution without Fe (Fe 0 treatment) or to the nutritive solution containing 50 μ M Fe (Fe 0 → Fe 50 treatment) for an additional week. Phenotypes were observed **A**, 24 h after the pretreatments by BABA or Fe 0 and **B**, 4 and **C**, 7 days after the plant transfer. In each frame, the upper line corresponds to the adaxial side and the lower line to the abaxial side of the leaves. Results are from one of three representative experiments.

Figure 3B and C. In summary, BABA-treated plants displayed macroscopic symptoms intermediate between those induced by a transient and a drastic iron deficiency.

BABA and Fe deficiency treatments

induce the accumulation of common metabolites.

A metabolomic analysis of leaves taken from plants 4 days after their transfer to the fresh nutritive solutions was performed using ultra-high-pressure liquid chromatography quadrupole

time-of-flight mass spectrometry (UHPLC-QTOFMS). We detected more than 2,900 features. Among them, 330, 333, and 663 accumulated differentially between control plants and plants treated with BABA or exposed to Fe 0 → Fe 50 or Fe 0 treatments, respectively (Supplementary Fig. S3). Six major compounds were tentatively identified as *p*-coumaroylagmatine, proline, indole-3-acetaldehyde, and three cyanidin 3-O glucosides—cyanidin 3-O-[2''-O-(xylosyl) 6''-O-(*p*-O-(glucosyl) *p*-coumaroyl) glucoside] 5-O-[6'''-O-(malonyl) glucoside], cya-

Fig. 4. Leaf metabolomic analysis of BABA- and Fe-deficiency-treated plants. After 4 weeks of hydroponic culture, plants were treated with 300 μ M BABA (BABA) or an equivalent volume of water (control) for 24 h and, then, were transferred to the nutritive solution without BABA for one supplementary week. For iron deficiency experiments, 4-week-old plants were treated for 24 h in the nutritive solution without Fe and then transferred either to the nutrient solution without Fe (Fe 0 treatment) or to the nutrient solution containing 50 μ M Fe (Fe 0 → Fe 50 treatment) for an additional week. **A**, Results of principal component analysis (PCA) after Pareto scaling on the MS data set. Analysis was performed on shoot tissues 4 days after the plant transfer. Peak lists were normalized by unit norm and subjected to PCA after Pareto scaling using the EZinfo software (Umetrics, Umea, Sweden). **B**, Relative quantification of the metabolites induced in Fe deficiency- or BABA-treated plants versus control plants. Metabolomic analyses were performed on shoot tissues, 4 days after the plant transfer, by ultra-high-pressure liquid chromatography quadrupole time-of-flight mass spectrometry. Each value represents the mean $\pm$ standard error of nine measurements (three replicates per experiment performed three times). Significant differences between the amounts of each metabolite were determined by a one-way analysis of variance followed by multiple comparison procedure with the least significant difference method, different letters signifying distinct statistical groups for each compound ($P < 0.05$).

nidin 3-O-[2''-O-(xylosyl)-6''-O-(*p*-coumaroyl) glucoside] 5-O-malonylglucoside, and cyanidin 3-(6''-*p*-coumaryl-2'''-sinapylsambubioside)-5-(6-malonylglucoside)—which are particular types of anthocyanins (Supplementary Table SI). More precisely, two *p*-coumaroylagmatine molecules, corresponding to two putative E and Z isomers named α and β , were identified.

The results of a principal component analysis performed on the MS data set after Pareto scaling are shown in Figure 4A. A clear separation between the control samples and the other treatments was observed. Notably, overlapping clusters were observed between BABA and Fe 0 $\rightarrow$ Fe 50 treatments. The relative quantifications of each compound in the four treatments are detailed in Figure 4B. Compared with control plants, a similar overaccumulation of the three anthocyanins ($C_{50}H_{57}O_{30}$, $C_{44}H_{47}O_{25}$, and $C_{55}H_{57}O_{29}$) was observed between BABA and Fe 0 $\rightarrow$ Fe 50 treatment. These three compounds deeply accumulated in the Fe 0 treatment. This result confirmed the phenotypic analysis shown in Figure 3. The isomer β of *p*-coumaroylagmatine ($C_{14}H_{21}N_4O_2$) and indole-3-acetaldehyde ($C_{10}H_9NO$) were more abundant in iron-deficient conditions (Fe 0 $\rightarrow$ Fe 50 and Fe 0 treatments). Proline ($C_5H_9NO_2$) is the only metabolite which accumulated only in response to Fe 0 treatment. Nevertheless, the quantification data relative to these three metabolites were not statistically different between BABA-treated plants and control plants. Concerning the isomer α of *p*-coumaroylagmatine, it strongly and similarly accumulated in both BABA and the complete iron-deficiency treatments.

Collectively, these results indicate that BABA triggered the accumulation of metabolites also accumulating in transient or permanent iron-deficiency conditions, further supporting the assumption that BABA affects iron homeostasis.

BABA treatment leads to a transient iron-deficiency response.

The phenotype analysis suggests that BABA triggers an iron-deficiency-like response. To further investigate this possibility, a comparative analysis of ferritin accumulation in leaves of plants grown in the hydroponic conditions described above was achieved by Western blotting. In control plants, the accumulation of ferritins increased with time, the strongest accumulation being observed 4 and 7 days after transfer to fresh

nutritive medium (Fig. 5). This variation in the level of ferritin accumulation is not surprising because ferritin rates were shown to be dependent on the leaf developmental stage and the life cycle (Petit et al. 2001). As expected, a drastic decrease in ferritin level was observed in the plants subjected to a permanent iron deficiency (Fe 0 treatment), thus confirming the efficiency of our experimental condition for inducing iron starvation. The decrease occurred from 24 h and was particularly drastic 4 and 7 days after the plant transfer to the iron-deficient medium. In comparison, the plants facing iron deficiency for only 24 h (Fe 0 $\rightarrow$ Fe 50 treatment) presented a stronger ferritin accumulation 4 days after the transfer in the nutritive solution containing iron. Seven days after the transfer, their levels of ferritin accumulation were similar to those detected in control plants. Remarkably, BABA treatment reduced the accumulation of ferritins to near the detection limit after the pretreatment. It is noteworthy that the decrease triggered by BABA pretreatment was more drastic than the one observed for iron deficiency. After transfer of the plants to fresh medium without BABA, ferritin accumulation increased to reach a level intermediate between the one of plants grown in permanent (Fe 0 treatment) and in transient (Fe 0 $\rightarrow$ Fe 50 treatment) iron-deficiency conditions.

To consolidate the assumption that BABA has a disturbing effect on iron homeostasis, the effect of BABA on the expression of the two iron-related genes *IRT1* and *FRO2* was investigated by qRT-PCR in roots and shoots of plants cultivated in BABA or iron-deficiency media (Fig. 6). Compared with control plants, the 24-h pretreatment in Fe-deficient medium led to a decrease in *IRT1* and *FRO2* transcript levels. Interestingly, a similar decrease was observed 24 h after the addition of BABA in the culture medium. Four days after the transfer, the levels of expression of *IRT1* and *FRO2* measured in BABA-treated plants were not statistically different from those measured in control plants, indicating that the effect of BABA seen after the 24-h pretreatment was transient. In contrast, the accumulation of both transcripts remained lower in Fe 0 $\rightarrow$ Fe 50- and Fe 0-treated plants, although to a lower extent in the Fe 0 $\rightarrow$ Fe 50 treatment. Seven days after the transfer, the transcripts decreased in all conditions and showed a similar level whatever the conditions. Taken together, these data show that

Fig. 5. Ferritin accumulation of the plants treated with BABA or grown in iron-deficient conditions. After 4 weeks of hydroponic culture, plants were treated with 300 μ M BABA (BABA) or an equivalent volume of water (control) for 24 h and, then, were transferred to the nutritive solution without BABA for one supplementary week. For iron deficiency experiments, 4-week-old plants were treated for 24 h in the nutritive solution without Fe and then transferred either to the nutrient solution without Fe (Fe 0 treatment) or to the nutrient solution containing 50 μ M Fe (Fe 0 $\rightarrow$ Fe 50 treatment) for an additional week. Accumulations of ferritins were detected by Western blotting in protein extracts prepared from leaves of plants **A**, pretreated for 24 h with BABA or Fe 0 and grown **B**, 4 and **C**, 7 days after transfer to the new medium. The Coomassie Blue (Coom.) and the Ponceau Red (Ponc.) stainings show equal loading and transfer, respectively. Results are from one of three representative experiments.

BABA treatment transiently affects the accumulation of *IRT1* and *FRO2* transcripts.

Globally, the changes in ferritin and in *IRT1* and *FRO2* transcript accumulations provide evidence that BABA-treated plants display iron homeostasis modifications typical of iron deficiency.

BABA-mediated iron deficiency is not correlated with a decrease in iron acquisition.

The effects of BABA on iron homeostasis reported above could be related to its ability to chelate and, therefore, remove all the iron present in the nutrient solution and available for plants. To investigate this possibility, we first checked whether the BABA-Fe complexes act as substrate for FRO. FRO activity was measured as reported by Yi and Gueriot (1996). For this purpose, plants were cultivated on plates in iron-sufficient conditions for 10 days and then transferred for 3 days in iron-sufficient or -deficient conditions. Next, roots were incubated with Fe(III)-EDTA or Fe(III)-BABA as a substrate for FRO activity. As expected, in the presence of Fe(III)-EDTA, FRO activity was strongly induced in plants cultivated in iron-deficient conditions (Fig. 7A). Interestingly, a similar FRO activity was measured in roots incubated with Fe(III)-BABA. Therefore, these data strongly suggest that Fe(III)-BABA complexes are, indeed, substrates for FRO and minimize the possibility that the effects of BABA on plant iron homeostasis are related to a reduction of iron availability in the nutrient solution. To strengthen this conclusion, we analyzed whether an increase of Fe availability in the nutrient solution could abolish the effects of BABA on iron homeostasis. For this purpose, plants were exposed to 350 μ M Fe-EDTA instead of 50 μ M and BABA concentration was kept at 300 μ M. The increase of iron concentration did not suppress the effects of BABA on the plant phenotype (Supplementary Fig. S4).

Classically, the iron-deficiency status can be due to either a decrease in root iron acquisition from the soil or from the nutritive solution, or to a reduction in iron availability in planta inde-

pendently of its import. Several processes might lead to a reduction in iron availability, including perturbations of the mechanisms involved in iron supply and distribution among the tissues or organs. A decrease in iron availability is usually accompanied by a stronger iron acquisition by the roots (Connolly et al. 2002; Vert et al. 2002). To determine the involvement of one of these two potential causes, root and shoot iron levels of the plants grown in the different conditions were measured by inductive coupled-plasma optical emission spectrometry (ICP-OES) (Fig. 7B and C). As expected, 4 and 7 days after the plant transfer, the iron concentrations in the roots and in the shoots of the Fe 0-treated plants were reduced compared with control plants. In BABA- and Fe 0 $\rightarrow$ Fe 50-treated plants, the root and shoot iron concentrations, respectively, were similar to those in control plants 24 h and 4 and 7 days after the transfer. Taken as a whole, these data show that the modifications induced by BABA treatment on the expression of ferritins, *IRT1*, and *FRO2* are not correlated to a decrease in iron acquisition by the roots.

BABA and Fe deficiency treatments modulate the expression of a similar set of 28 genes.

We compared the results of two transcriptomic analyses obtained in *A. thaliana* shoots in response to BABA (Tsai et al. 2011) and Fe deficiency (Schuler et al. 2011). In the first study, plants were soil drenched with BABA at a final concentration of 200 μ M, or with water, for 2 days 22 h before RNA sample collection. In the second one, gene expression of L3/L4 rosette leaves of wild-type plants grown for 1 week under sufficient or deficient Fe medium solution were analyzed. We compared the list of the genes modulated in response to Fe deficiency (2,872 genes) with the list of the 55 BABA-modulated genes. The comparison was reduced to 46 genes because nine of the 55 BABA-modulated genes were absent in the microarray used by Schuler and colleagues (2011). Interestingly, we found that 28 genes were commonly modulated by both treatments (Table 1), thus representing 61% of the BABA-dependent genes. More precisely, among those 46 genes, 22

Fig. 6. Impact of BABA and Fe deficiency on the expression of iron-related genes. **A**, *IRT1* and **B**, *FRO2* transcript accumulation were measured in roots. After 4 weeks of hydroponic culture, plants were treated with 300 μ M BABA (BABA) or an equivalent volume of water (control) for 24 h and, then, were transferred to the nutritive solution without BABA for one supplementary week. For iron deficiency experiments, 4-week-old plants were treated for 24 h in the nutritive solution without Fe and then transferred either to the nutrient solution without Fe (Fe 0 treatment) or to the nutrient solution containing 50 μ M Fe (Fe 0 $\rightarrow$ Fe 50 treatment) for an additional week. Measurements of *IRT1* and *FRO2* transcripts accumulation were performed by qRT-PCR. Data are expressed as mean normalized expression relative to the appropriate housekeeping gene *AtYLS8* (arbitrary unit) and are the means of triplicates ($\pm$ standard error) from one representative experiment of three independent experiments. Significant differences between the treatments were determined by a one-way analysis of variance followed by multiple comparison procedure with the least significant difference method, different letters signifying distinct statistical groups ($P < 0.05$).

were induced by both treatments, 4 were induced by BABA but repressed by Fe deficiency and two were repressed by BABA but induced by Fe deficiency.

To complete this comparison, we used the tool “signature” of Genevestigator (Hruz et al. 2008). Among the 166 nutrient perturbations listed in the software, profiles from two transcriptional analyses studying the mutants in iron homeostasis *nas4x-1* (versus Col-0 in Fe sufficiency conditions) and *bhlh100/bhlh101* (versus Col-0 in Fe deficiency conditions) are among the most similar to the gene profile obtained after BABA treatment by Tsai and associates (2011), with relative similarity of 1.292 and 1.227 (Euclidean distance), respectively (Supplementary Fig. S5). The *nas4x-1* mutant is affected in the expression of the four *NAS* genes present in the *A. thaliana* genome and contains very low level of NA. It displays leaf chlorosis, reduced Fe content in the flowers and seed, sensitivity to

Fe deficiency, and disturbances in Fe homeostasis such as up-regulation of *IRT1* and *FRO2* (Klatte et al. 2009). As *nas4x-1*, the double mutant *bhlh100/bhlh101* is hypersensitive to Fe deficiency. Indeed, the transcription factors *BHLH100* and *BHLH101* function as key regulators of Fe deficiency responses independently of FIT1. They have been shown to regulate genes involved in the distribution of iron within the plant (Sivitz et al. 2012).

These data highlight the idea that BABA treatment and Fe deficiency probably share common signaling pathways.

Iron deficiency protects the plants against *B. cinerea*.

The virulence of certain microbial plant pathogens is linked to their ability to produce siderophores that act in trapping the host iron in order to use it for their own development. For example, pyoverdine, a siderophore produced by *P. fluorescens*, induces

Fig. 7. Effects of Fe(III)-EDTA and Fe(III)-BABA complexes on root ferric-chelate reductase (FRO) activity and **B**, shoot and **C**, root iron concentration in plants exposed to BABA or grown in iron-deficient conditions. **A**, For measurement of FRO activity, plants were cultivated 10 days on plates supplemented with 50 μM Fe-EDTA and transferred for 3 days under iron-sufficient (Fe 50 μM; roots + Fe) or iron-deficient (Fe 0 μM + 300 μM Ferrozine; roots - Fe) conditions. Then, roots were treated for 20 min with Fe(III)-EDTA or Fe(III)-BABA as a substrate. Each value represents the mean of triplicates (± standard error) from one representative experiment of three independent experiments. Significant differences between the treatments were determined by a one-way analysis of variance (ANOVA) followed by multiple comparison procedure with the least significant difference method, different letters signifying distinct statistical groups ($P < 0.01$). **B**, Shoot and **C**, root iron concentrations of the plants treated with BABA or grown in iron-deficient conditions. After 4 weeks of hydroponic culture, plants were treated with 300 μM BABA (BABA) or an equivalent volume of water (control) for 24 h and, then, were transferred to the nutritive solution without BABA for one supplementary week. For iron deficiency experiments, 4-week-old plants were treated for 24 h in the nutritive solution without Fe and then transferred either to the nutrient solution without Fe (Fe 0 treatment) or to the nutrient solution containing 50 μM Fe (Fe 0 → Fe 50 treatment) for an additional week. Measurement of iron concentrations was performed by inductive coupled-plasma optical emission spectrometry. Each value represents the means ± standard error of nine measurements (three replicates per experiment performed three times). Significant differences between the treatments were determined by a one-way ANOVA followed by multiple comparison procedure with the least significant difference method, different letters signifying distinct statistical groups ($P < 0.05$). FW = fresh weight and DW = dry weight.

defense reactions in plants (Van Loon et al. 2008). Therefore, a decrease in the iron availability due to siderophores could create a warning signal activating the plant immune system. Based on this hypothesis, one can assume that the potentialization of plant defense reactions induced by BABA is partly dependent on its capacity to disturb plant iron homeostasis. Moreover, it has been shown that iron deficiency protects *A. thaliana* plants against *D. dadantii* and *B. cinerea* infections (Kieu et al. 2012).

We compared the effects of BABA-induced resistance against the fungal pathogen *B. cinerea*, which was first shown by Zimmerli and associates (2001), to the putative resistance induced by iron deficiency. Plants grown in hydroponic conditions as described below were immediately subjected to *B. cinerea* infection after 24 h of pretreatment with BABA or iron deficiency. Then, the diameter of the necrotic lesions was measured 3 days postinoculation. Compared with the control,

Table 1. List of the 28 genes regulated in response to BABA versus water and to Fe deficiency (–Fe) versus Fe sufficiency (+Fe)^a

AGI Number	Name	Fold-change in response to	
		BABA vs. water	–Fe vs. +Fe
At3g11340	UDP-DEPENDENT GLYCOSYLTRANSFERASE 76B1, UGT76B1	4.96	5.34
At3g29250	SHORT-CHAIN DEHYDROGENASE REDUCTASE 4, ATSDR4, SDR4	3.08	5.41
At4g35180	Lys/His transporter 7, LHT7	2.77	2.99
At3g52430	ARABIDOPSIS PHYTOALEXIN DEFICIENT 4, ATPAD4	2.56	2.14
At5g64000	3'(2'),5'-bisphosphate nucleotidase	2.47	5.92
At5g18470	Curculin-like (mannose-binding) lectin family protein	2.46	2.05
At1g10340	Ankyrin repeat family protein	2.34	3.43
At3g16530	Lectin like protein (whose expression is induced upon treatment with chitin oligomers)	2.32	2.09
At5g51440	HSP20-like chaperones superfamily protein	2.28	9.16
At3g57260	β-1,3-GLUCANASE 2, BG2, BGL2, PATHOGENESIS-RELATED PROTEIN 2, PR-2	2.28	4.95
At5g60800	Heavy metal transport/detoxification superfamily protein	2.27	6.23
At5g10380	ATRIN1	2.26	2.11
At4g04490	CRK36, CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 36	2.23	19.81
At4g04540	CRK39, CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 39	2.19	2.66
At4g04500	CRK37, CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 37	2.17	6.96
At4g25110	METACASPASE 2, ATMC2	2.16	2.80
At3g21080	ABC transporter-related	2.10	4.87
At4g18250	Receptor serine/threonine kinase, putative	2.09	3.10
At1g51800	IMPAIRED OOMYCETE SUSCEPTIBILITY 1, IOS1	2.09	2.55
At5g24530	DOWNY MILDEW RESISTANT 6, DMR6	2.04	4.36
At4g21120	AMINO ACID TRANSPORTER 1, AAT1; CATIONIC AMINO ACID TRANSPORTER 1, CAT1	2.02	2.78
At1g74710	ISOCHORISMATE SYNTHASE 1, ATICS1; SALICYLIC ACID INDUCTION DEFICIENT 2, SID2	2.02	4.30
At1g59950	NAD(P)-linked oxidoreductase superfamily protein	2.49	0.40
At2g34930	Disease resistance family protein / LRR family protein	2.21	0.38
At3g46900	COPPER TRANSPORTER 2, COPT2	2.13	0.40
At4g36010	Pathogenesis-related thaumatin superfamily protein	2.09	0.27
At2g28190	COPPER/ZINC SUPEROXIDE DISMUTASE 2, CSD2	0.23	2.01
At1g12520	COPPER CHAPERONE FOR SOD1, ATCCS	0.33	2.73

^a Results of two transcriptomic analyses obtained in *Arabidopsis thaliana* shoots exposed to BABA (Tsai et al. 2011) or Fe deficiency (Schuler et al. 2011) were compared. Genes showing a minimum induction of two- (dark gray) or a minimum repression of 0.5-fold (light gray) were selected ($P < 0.05$, t test, $n = 3$ per group).

Fig. 8. *Botrytis cinerea* symptom development in the leaves of plants pretreated with BABA or grown in iron-deficient conditions. After 4 weeks of hydroponic culture, plants were treated with 300 μ M BABA (BABA) or an equivalent volume of water (control) for 24 h and, then, were transferred to the nutritive solution without BABA for one supplementary week. For iron deficiency experiments, 4-week-old plants were treated for 24 h in the nutritive solution without Fe and then transferred either to the nutrient solution without Fe (Fe 0 treatment) or to the nutrient solution containing 50 μ M Fe (Fe 0 $\rightarrow$ Fe 50 treatment) for an additional week. Disease resistance of the plants was tested against *B. cinerea* isolate BMM. Leaves were inoculated after the plant transfer. Five leaves from 12 plants were used for each treatment. Disease resistance was scored by measuring the necrotic lesion diameters 3 days postinoculation (dpi). Histograms show lesion diameter (d) classification according to their size in four classes ($d \leq 2$ mm; $2 \text{ mm} < d \leq 4$ mm; $4 \text{ mm} < d \leq 6$ mm; and $d \geq 6$ mm). Results are from one representative experiment of three independent experiments.

under BABA, Fe 0 → Fe 50, and Fe 0 conditions, plants were more resistant to infection. In particular, we did not observe lesions showing diameters bigger than 6 mm in these three conditions whereas this category was prominent in control plants (47% of the lesions) (Fig. 8).

The decreased symptoms induced by *B. cinerea* in BABA-, Fe 0 → Fe 50-, and Fe 0-treated plants led us to measure the impact of the pretreatments on the expression of the two defense-related genes *PR1* and *PDF1.2*. Shoots of plants grown in hydroponic conditions were sprayed with *B. cinerea* spores after 24 h of pretreatment with BABA or iron deficiency and the tissues were harvested 48 h after. Although BABA had no effect on the expression of the genes in mock treatment, the transient and the drastic Fe deficiency conditions both induced their activation. In response to *B. cinerea*, the expressions of *PR1* and *PDF1.2* are higher in BABA-, Fe 0 → Fe 50-, and Fe 0-treated plants compared with control plants. Notably, the effects of Fe 0 → Fe 50 and Fe 0 treatments were slightly higher in response to *B. cinerea* than in response to mock treatment for *PR1* and no additive effect was observed for *PDF1.2* between these two conditions (Fig. 9).

DISCUSSION

Our present investigation contributes to a better understanding of the still largely unknown processes inherent in the effects of BABA, a molecule able to potentiate plant defense mechanisms and to protect plants against a variety of biotic and abiotic stresses.

An *in silico* structural analysis of BABA suggested that it might display Fe chelation properties. Indeed, the carboxylic and amino groups of each molecule of BABA are both able to coordinate Fe, the formation of the complex being possible in terms of energy and spatial arrangement. We propose a model in which three molecules of BABA coordinate one Fe³⁺ ion. This chelating property has already been shown for other amino acids such as glycine, with two amino acids forming a complex with one ion of iron, copper, or zinc (Mandado and Cordeiro 2010; Pizarro et al. 2002).

The Fe chelating properties of BABA led us to examine its impact on in planta Fe homeostasis. We observed that a pretreatment with BABA of *A. thaliana* plants grown in soil or hydroponic conditions modulates the expression of genes or proteins involved in Fe sequestration and transport. Drastic decreases in *FRO2* and *IRT1* transcript and ferritin accumulations were observed. These decreases were also observed in the plants facing iron deficiency. Analysis of the BABA-pretreated plants transferred in a medium deprived of BABA for one additional week indicates that its drastic effects on the accumulation of the transcripts and proteins of interest were transitory. Interestingly, these accumulation profiles were relatively similar to those observed in plants subjected to a transient Fe deficiency. These results suggest that BABA pretreatment induces in *A. thaliana* a series of events characteristic of Fe deficiency. We checked that these effects were not related to the removal of the iron present in the nutritive solution that BABA might cause through its Fe-chelating properties.

The ability of BABA to affect iron homeostasis was further confirmed by the phenotypical analysis showing violet pigmentation of the lower sides of the leaves in both BABA-treated plants and plants exposed to a transient or permanent Fe deficiency. An increase in the total foliar anthocyanin content in response to BABA associated with the activation of chalcone synthase and dihydroflavonol-4-reductase activities was previously shown by Wu and associates (2010). In the present study, we identified three anthocyanins whose production was stimulated by BABA. These flavonoid pigments are also

synthesized in response to other stresses such as desiccation or wounding or during pathogen attacks and were shown to shield tissues by preventing the deleterious effects of reactive oxygen species and free radicals (Gould 2004). Importantly, the metabolomic analysis also revealed a significant production of E or Z *p*-coumaroylglutamine in BABA and Fe 0 treatments. The production of this hydroxycinnamic acid amide was previously shown to be induced in several plant species in response to pathogen attack such as in *A. thaliana*–*Alternaria brassicicola* (Muroi et al. 2009) and barley–*Erysiphe graminis* f. sp. *hordei* (von Röpenack et al. 1998) interactions. These data further confirm that BABA and iron deficiency trigger common processes, including defense-related mechanisms. More generally, the finding that 61% of the BABA-modulated genes identified in the investigation of Tsai and associates (2011) were also modulated by Fe deficiency (Schuler et al. 2011) highlights the

Fig. 9. Impact of BABA and Fe deficiency on the expression of defense-related genes. **A**, *PR1* and **B**, *PDF1.2* transcript accumulations were measured in shoots. After 4 weeks of hydroponic culture, plants were treated with 300 μM BABA (BABA) or an equivalent volume of water (control) for 24 h and, then, were transferred to the nutritive solution without BABA for one supplementary week. For iron deficiency experiments, 4-week-old plants were treated for 24 h in the nutritive solution without Fe and then transferred either to the nutritive solution without Fe (Fe 0 treatment) or to the nutritive solution containing 50 μM Fe (Fe 0 → Fe 50 treatment) for an additional week. Leaves were sprayed following the plant transfer, and the shoots were harvested 48 h after. Measurements of *PR1* and *PDF1.2* transcript accumulations were performed by quantitative reverse-transcriptase polymerase chain reaction. Data are expressed as mean normalized expression relative to the appropriate housekeeping gene *AtYLS8* (arbitrary unit) and are the means of triplicates (± standard error) from one representative experiment of three independent experiments. Significant differences between the treatments were determined by a one-way analysis of variance followed by multiple comparison procedure with the least significant difference method, different letters signifying distinct statistical groups ($P < 0.05$).

possibility that BABA activates signaling pathways used by plants facing Fe starvation.

The comparative analyses of iron concentrations between BABA-treated plants and plants facing drastic Fe deficiency revealed that BABA did not induce a decrease in Fe concentration. Therefore, the BABA-induced transient Fe deficiency response deduced from the accumulation profiles of the transcripts and proteins related to Fe homeostasis is not caused by a reduction in Fe uptake. Rather, it might reflect a decrease in Fe availability. This statement raises the question of how BABA modulates Fe availability. First, it is conceivable that BABA is rapidly taken up by the roots and accumulates in the leaves. Using ^{14}C -BABA, it was indeed shown in lettuce plants that the amino acid is taken up within 2 h by the roots and, then, is transported from 18 h in the cotyledons before being incorporated in all the tissues by 45 h (Cohen et al. 2010). This systemic translocation was also shown in other species, including tomato (Cohen and Gisi 1994) and *A. thaliana* (Jakab et al. 2001; Návarová et al. 2012). Based on these findings, we assume that once internalized in plants, BABA may trigger an Fe deficiency via its capacity to bind Fe. The finding that the Fe(III)-BABA stability constant was higher than those of Fe(III)-citric acid, a physiological complex involved in iron chelation and distribution, supports this hypothesis. Due to the transient effects of BABA on iron homeostasis, it is conceivable that BABA-treated plants set up Fe acquisition strategies promoting Fe recovery from the BABA-Fe complexes. Alternatively, BABA could also be taken up by the roots as BABA-Fe complexes. In this scenario, Fe deficiency might be related to the low Fe availability of the Fe bound to BABA. However, the observation that the BABA-Fe complexes act as substrates of the root ferric-chelate reductase clearly minimizes this hypothesis.

Our results also raise the question of the functional link between BABA effects on Fe homeostasis and its capacity to potentiate plant defense mechanisms against pathogen microorganisms. The chelation of Fe of the hosts by siderophores secreted by pathogen microorganisms should constitute signals triggering an immune response in the infected plants (Segond et al. 2009). For instance, Aznar and associates (2014) recently showed that the iron scavenger activity of the bacterial siderophore deferrioxamine triggers immunity in *A. thaliana* through perturbation of iron homeostasis. To test this hypothesis, we compared the impacts of Fe deficiency with those of BABA treatment in *A. thaliana* plants infected by the fungus *B. cinerea*. Our analysis pointed out that the transient Fe deficiency and BABA treatments decrease the sensitivity of *A. thaliana* plants to *B. cinerea* with the same intensity. Disease reduction in iron-starved plants was also observed with the bacterial plant pathogen *D. dadantii* in *A. thaliana* (Kieu et al. 2012), indicating that the plant nutritional iron status affects the response to several pathogens, highlighting a more general process. One possible explanation is that the lack of bioavailable Fe resulting from Fe deficiency reduces the Fe availability for the pathogen and, therefore, weakens its development. By chelating Fe in planta, BABA could mimic this process. Another explanation is that Fe deficiency could place the plant in a defensive waking state via the induction of defense reactions. In line with this proposition, we showed that iron deficiency provokes the transcript accumulation of genes coding key defense proteins. Therefore, as stated above, BABA and Fe deficiency treatments probably induce common defense-related pathways. Therefore, the chelation of endogenous iron by BABA leading to Fe deficiency could represent a warning signal, putting plants in a defensive state. In this scenario, BABA could mimic the action of a microbial siderophore. However, although the perturbation of iron homeostasis could account for BABA

effects, the mode of action of BABA may not be directly comparable with that triggered by iron deficiency or microbe siderophores and clearly involves iron-independent processes. Accordingly, in contrast to BABA treatment, we observed that Fe deficiency by itself triggered a significant induction of *PR1* and *PDF1.2* and, at least in the case of *PDF1.2*, did not induce a priming mechanism in response to *B. cinerea*.

Conclusion.

Our study demonstrates that BABA induces a transient Fe deficiency response in *A. thaliana* plants, probably due to disturbances in the availability of the metal. These changes could be the consequences of the in planta Fe chelation by the amino acid. We also bring evidence suggesting that Fe deficiency could place the plant in a defensive waking state, reinforcing its resistance. Therefore, the transient Fe deficiency induced by BABA could account for its protective effects against pathogens but probably not for BABA priming effects. In short, this survey opens up new horizons for the understanding of BABA-induced resistance.

MATERIALS AND METHODS

Plant material and growth conditions.

Experiments were performed using the wild-type *A. thaliana* accession Columbia (Col-0).

For soil experiments, plants were cultivated in a commercial soil (Jiffy-7, Moerdijk, The Netherlands). For hydroponic culture, seed were surface sterilized by immersion in a solution containing 1.2% bleach and 50% EtOH for 5 min, rinsed three times with 100% EtOH, air dried, and placed for 3 days at 4°C in the dark in 0.15% agar. Then, they were sown in seed holders filled with 0.75% agar and put in a cover placed above the nutrient solution container (Araponics, Liege, Belgium). The nutritive medium (0.25 mM $\text{Ca}(\text{NO}_3)_2$, 0.5 mM KNO_3 , 1 mM KH_2PO_4 , 1 mM MgSO_4 , 50 μM H_3BO_3 , 19 μM MnCl_2 , 10 μM ZnCl_2 , 1 μM CuSO_4 , 0.02 μM Na_2MoO_4 , and 50 μM Fe-Na-EDTA) was changed once a week. Plants were allowed to grow in a climate-controlled growth chamber for 4 weeks before the treatments in the following conditions: 10-h day (200 $\mu\text{E m}^{-2} \text{s}^{-1}$ light intensity, 20°C) and 14-h night (18°C), with 70% relative humidity.

Chemicals.

All chemicals were purchased from Sigma-Aldrich (St. Louis), unless otherwise stated.

Treatments.

BABA treatment. For plants grown for 4 weeks in soil, Jiffy clods were drenched with a 300 μM BABA solution 1 day prior to harvesting samples for ferritin quantification. For plants cultivated for 4 weeks in hydroponic systems, roots were washed with fresh nutrient solution without Fe-Na-EDTA. Then, the plants were treated for 24 h in a fresh nutrient solution with 300 μM BABA (BABA treatment) or with an equivalent volume of water (control treatment). After root washing with the nutrient solution without Fe-Na-EDTA to remove BABA, they were cultivated in freshly prepared nutrient solution for an additional week.

Iron-deficiency treatments. After 4 weeks of hydroponic culture, roots were washed and then treated for 24 h in a fresh nutrient solution without Fe-Na-EDTA. After root washing with the nutrient solution without Fe-Na-EDTA, plants were separated into two sets: half were put in the nutrient solution without Fe-Na-EDTA (Fe 0 treatment) and half were grown in the nutrient solution containing 50 μM Fe-Na-EDTA (Fe 0 → Fe 50 treatment) for an additional week.

qRT-PCR analyses.

Root and shoot tissues were collected separately after the treatments and immediately frozen in liquid nitrogen before total RNA extraction using Trizol reagent, according to the manufacturer's instructions (Invitrogen, Paisley, UK). A DNase (Promega Corp., Madison, WI, U.S.A.) treatment was performed on 500 ng of total RNA to prevent genomic DNA contamination. RNA samples were subsequently used for reverse transcription (ImpromII Reverse Transcriptase kit; Promega Corp.) with anchored oligo(dT15) (Promega Corp.) and 0.4 mM deoxynucleotide triphosphates. The resulting cDNAs were subjected to a twofold dilution with water, and 1 µl of each cDNA sample was assayed by qPCR in a LightCycler using GoTaq qPCR Master Mix (Promega Corp.). Expression levels were calculated relatively to the housekeeping gene *AtYLS8* (*At5g08290*) (Czechowski et al. 2005; Han et al. 2013) using the relative standard curve method. For each sample, target quantity of the gene of interest was determined by interpolating the value from the standard curve made from a cDNA pool which enabled us to take into consideration the efficiency of amplification. The value was then divided by the target quantity of the housekeeping gene.

Primer sequences were as follows: YLS8-fw: 5'-GTCATTTATCTGGTGGACATCACT-3' and YLS8-rev: 5'-CCGAAACAGTAAGCAAAAAC-3', FER1-fw: 5'-CAGACTACATCACCCAGCTAAGGA-3' and FER1-rev: 5'-TCTGGTCGAAATGCCAACTC-3', NAS4-fw: 5'-TTGTGTTCTTGGCTGCTCTTG-3' and NAS4-rev: 5'-TGCTCGACCACCTTAACCTCCT-3', IRT1-fw: 5'-GCTTCTTTTCGCGGTAACA-3' and IRT1-rev: 5'-AGTTGATAGAGCGATCCCTAACG-3', FRO2-fw: 5'-CGATCGTTTCCTTCGGTTTC-3' and FRO2-rev: 5'-AATCCGAGCAGCGAGCAA-3', PR1-fw: 5'-TCTTCCCTCGAAAGCTCAAGA-3' and PR1-rev: 5'-GTGCCTGGTTGTGAACCTTA-3', PDF1.2-fw: 5'-CACCGGCAATGGTGGAA-3', and PDF1.2-rev: 5'-CCCTGACCATGTCCCACT-3'.

FRO activity assay.

Root FRO activities were determined by spectrophotometry, as described previously by Yi and Guerinot (1996). Plants were grown for 10 days on petri plates supplemented with 50 µM Fe-EDTA and transferred for 3 days on iron-sufficient (50 µM Fe) or iron-deficient (0 µM Fe + 300 µM Ferrozine) media, as already reported (Yi and Guerinot 1996). Then, the entire root system of seven plants was submerged in a solution of 10 mM morpholineethanesulfonic acid, pH 5.5, containing 100 µM Fe(III)-EDTA and 300 µM Ferrozine or 100 µM Fe(III)-300 µM BABA and 300 µM Ferrozine. After 20 min in the dark at room temperature, roots were removed and the absorbance of the solution was determined at 562 nm. Concentration of Fe(II)-Ferrozine was calculated using a molar extinction coefficient of 28.6 mM⁻¹ cm⁻¹ (Gibbs 1976).

Immunodetection of ferritins.

Total protein extracts were prepared from samples as previously described (Lobréaux et al. 1992). Immunodetection of ferritins was performed using a rabbit polyclonal antiserum raised against purified AtFER1 protein (Dellagi et al. 2005) recognizing the four ferritin subunits (Ravet et al. 2009). As loading controls, the corresponding protein gel and the nitrocellulose membrane were respectively stained with Coomassie Blue or Ponceau Red. For all treatments, a similar band corresponding to the same proteins was chosen at random to show equal loading and transfer.

Metabolomic analyses.

Shoot tissues were collected after the treatments and immediately frozen in liquid nitrogen before being ground to a fine

powder. Powder (100 mg) was transferred into a microcentrifuge tube. Then, 0.5 ml of extraction solvent consisting of a mixture of methanol, water, and formic acid (80:19.5:0.5, vol/vol) as well as a few glass beads (2 mm in diameter) were added to the tubes, which were immediately vortexed, shaken for 3 min at 30 Hz in a tissue lyser (Retsch MM300, Haan, Germany), and centrifuged at 14,000 × g for 3 min. The supernatant was transferred into an appropriate vial for analysis.

UHPLC-QTOFMS metabolomic analyses were performed on a Synapt G2 QTOFMS (Waters, Milford, MA, U.S.A.) interfaced to an Acquity UPLC system (Waters) through an electrospray ionization source. In a first step, both positive and negative ion modes were tested, and positive ion mode was further selected for subsequent analyses because more discriminant signals were revealed in this mode. The MS was operated over 85 to 1,200 Da in the so-called MSE mode using scan times of 0.2 s at both low (4 eV) and high (10 to 30 eV ramp) collision energies. Argon was used as collision gas at a flow rate of 2.2 ml min⁻¹ and a pressure of 7.2 × 10⁻³ mbar. To obtain accurate mass measurements, external and internal calibrations were applied by infusing solutions of sodium formate (0.5 mM) and leucine-enkephalin (400 ng ml⁻¹), respectively, at a flow rate of 10 µl min⁻¹. The source parameters were as follows: source temperature = 120°C; capillary voltage = 2,800 V; cone voltage = 25 V; desolvation gas flow and temperature = 800 liters h⁻¹ and 330°C, respectively; and cone gas flow = 20 liters h⁻¹. The UHPLC separation involved an Acquity BEH C18 column (2.1 by 50 mm, 1.7-µm particle size) (Waters) and mobile phases consisting of 0.05% formic acid in water as solvent A and 0.05% formic acid in acetonitrile as solvent B. The following gradient program was applied: 2 to 30% B in 3.0 min, 30 to 100% B in 4.0 min, holding at 100% B for 2.0 min, and reequilibration at 2% B for 1.5 min. The flow rate was set to 0.4 ml min⁻¹. The column temperature was maintained at 30°C. The injection volume was of 2.5 µl. The entire LC-MS system was controlled by Masslynx V.4.1.

UHPLC-QTOFMS data were processed with Markerlynx XS software (Waters). Mass signals were extracted and aligned from 0 to 6.5 min using the following parameters: mass range 85 to 1,200 Da, mass window 0.02 Da, retention time window 0.06 min, marker intensity threshold 400 counts, automatic calculation of peak width and peak-to-peak baseline noise, noise elimination disabled, and deisotoping enabled.

Determination of Fe concentrations.

The concentrations of Fe were determined both in the roots and in the shoots by ICP-OES (ICB-UMR 6303/Université De Bourgogne, Dijon, France).

Plant inoculation with *B. cinerea*.

B. cinerea BMM strain was grown on potato dextrose agar plates (Duchefa, Haarlem, The Netherlands) at 39 g liter⁻¹ and 16°C for 10 days (10 h of day and 14 h of night) until sporulation. Spores were harvested in sterile water and filtered through glass wool to remove hyphae. Then, they were diluted in PDB medium (BD Biosciences, San Jose, CA, U.S.A.) at 6 g liter⁻¹ for inoculation or spray. For quantification of lesion size, plants were inoculated by applying 6-µl droplets of spore suspensions at a concentration of 5 × 10⁴ spores ml⁻¹ to the upper surface of the leaves. For measurement of defense gene expression, leaves were sprayed with a solution of 2 × 10⁵ spores ml⁻¹. The inoculated or sprayed plants were incubated at 100% relative humidity and 20°C with a 12-h photoperiod. Symptom development was scored by measuring the maximal lesion diameter. Control inoculation or spray were performed with PDB culture medium only (mock treatment).

Electrochemical measurements.

All electrochemical experiments were performed with a μ -AUTOLAB III (Metrohm, Herisau, Switzerland) potentiostat connected to a PC and the data collected were analyzed using Nova 1.7 software. Rotating disk electrode voltammetry was carried out by means of a three-electrode configuration: a platinum wire as counter electrode, a saturated calomel electrode (SCE) as reference, and a Pt rotating disk electrode (2 mm in diameter, EDI 101; Radiometer, Copenhagen, Denmark) as working electrode. The rotation speed was controlled with a speed control unit (CTV 101; Radiometer). Potentials are reported versus SCE. Voltammograms were recorded at 1,500 rpm.

The Fe^{3+} solutions were prepared by adding, in a 25-ml volumetric flask, 1.25×10^{-4} mol of Fe(III) chloride hexahydrate and 0, 1, 2, or 3 equivalents of BABA.

The Fe^{3+} solutions (5×10^{-3} M) were deoxygenated with argon and a positive overpressure of argon was maintained above the electrolyte during the entire measurement performed at room temperature.

UV-visible spectroscopy.

The spectra were recorded with a UV-visible diode array spectrometer (KINSPEC II/MMS-16 VIS, BIOLOGIC, Grenoble, France). The 150-W Xe lamp and the spectrometer were connected to the cell (optical length = 1 mm) by light fibers.

Statistics.

In order to detect significant differences between the treatments, one-way analysis of variance was performed using the statistical program STATGRAPHICS (release 5.1; Manugistic Inc., Rockville, MD, U.S.A.) followed by a multiple comparison procedure with the Fisher's least significant difference method ($P < 0.05$). For the Western blotting anti-ferritin and NAS4 and FER1 transcript accumulations in the plants grown in soil, significant differences from control plants were determined by the Student's t test (* indicates $P < 0.05$).

ACKNOWLEDGMENTS

This work was supported by the University of Burgundy, the Burgundy State (PARI AGRAL 8 project), and the French Ministry of Agriculture.

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