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Original Article

Airborne signals from *Trichoderma* fungi stimulate iron uptake responses in roots resulting in priming of jasmonic acid-dependent defences in shoots of *Arabidopsis thaliana* and *Solanum lycopersicum*

Ainhoa Martínez-Medina[1,2], Saskia C.M. Van Wees[1] & Corné M.J. Pieterse[1]

[1]*Plant-Microbe Interactions, Department of Biology, Utrecht University, 3584 CH Utrecht, The Netherlands and* [2]*Molecular Interaction Ecology, German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Institute of Ecology, Friedrich Schiller University, Leipzig 04103, Germany*

ABSTRACT

Root colonization by *Trichoderma* fungi can trigger induced systemic resistance (ISR). In Arabidopsis, *Trichoderma*-ISR relies on the transcription factor MYB72, which plays a dual role in the onset of ISR and the activation of Fe uptake responses. Volatile compounds (VCs) from rhizobacteria are important elicitors of *MYB72* in Arabidopsis roots. Here, we investigated the mode of action of VCs from *Trichoderma* fungi in the onset of ISR and Fe uptake responses. VCs from *Trichoderma asperellum* and *Trichoderma harzianum* were applied in an *in vitro* split-plate system with Arabidopsis or tomato seedlings. Locally, *Trichoderma*-VCs triggered *MYB72* expression and molecular, physiological and morphological Fe uptake mechanisms in Arabidopsis roots. In leaves, *Trichoderma*-VCs primed jasmonic acid-dependent defences, leading to an enhanced resistance against *Botrytis cinerea*. By using Arabidopsis micrografts of VCs-exposed rootstocks and non-exposed scions, we demonstrated that perception of *Trichoderma*-VCs by the roots leads to a systemic signal that primes shoots for enhanced defences. *Trichoderma*-VCs also elicited Fe deficiency responses and shoot immunity in tomato, suggesting that this phenomenon is expressed in different plant species. Our results indicate that *Trichoderma*-VCs trigger locally a readjustment of Fe homeostasis in roots, which links to systemic elicitation of ISR by priming of jasmonic acid-dependent defences.

Key-words: induced systemic resistance; iron deficiency; microbial volatile compounds; mutualistic fungi; plant-microbe interaction; tomato.

INTRODUCTION

Species from the genus *Trichoderma* are among the most commonly isolated saprotrophic fungi in both natural and agricultural ecosystems (Druzhinina *et al.* 2011). They are often found associated with plant roots, where sugars excreted by the host root deliver important carbon sources (Vargas *et al.* 2009, 2011). In turn, *Trichoderma* fungi provide essential services to the plant, as they modulate processes related to nutrient uptake and recycling, degradation of phytotoxic compounds, control of deleterious soil microbiota and stimulation of root and shoot development (Harman *et al.* 2004; Harman 2011; Martinez-Medina *et al.* 2014). Moreover, selected *Trichoderma* strains can boost plant defences in systemic tissues, rendering the entire plant more resistant against a variety of pathogens and pests. This phenomenon is called induced systemic resistance (ISR) and can be triggered by diverse beneficial microbes, including plant growth-promoting rhizobacteria and mycorrhizal fungi (Van Wees *et al.* 2008; Jung *et al.* 2012; Pieterse *et al.* 2014). ISR triggered by beneficial microbes, like *Trichoderma* fungi, is generally associated with a 'sensitization' of the plant's immune system resulting in enhanced activation of jasmonic acid (JA)-regulated gene expression and an enhanced deposition of callose-rich papillae at the sites of pathogen entry (Segarra *et al.* 2009; Martinez-Medina *et al.* 2013, 2017). This phenomenon is known as defence priming and provides the plant with a cost-effective mechanism of protection against pathogens and pests (Conrath *et al.* 2015; Martinez-Medina *et al.* 2016).

In the roots of Arabidopsis (*Arabidopsis thaliana*), initiation of ISR by *Trichoderma asperellum* strain T-34 is regulated by the R2R3-type MYB transcription factor MYB72 (Segarra *et al.* 2009). Upon colonization by *T. asperellum* T-34, *MYB72* gene expression is activated in the roots. Moreover, Arabidopsis *myb72* mutants are impaired in their ability to express *Trichoderma*-ISR against different shoot pathogens, indicating that this root-specific transcription factor is essential for the onset of ISR. Interestingly, ISR triggered by the rhizobacterium *Pseudomonas simiae* (formerly known as *Pseudomonas fluorescens* WCS417; Berendsen *et al.* 2015) is also associated with the activation of *MYB72* in the roots and dependent on MYB72 functioning (Van der Ent *et al.* 2008), suggesting that this transcription factor is a node of convergence in the ISR signalling pathway triggered by different beneficial microbes.

Correspondence: Ainhoa Martínez-Medina and Corné M.J. Pieterse. e-mail: ainhoa_martinez.medina@idiv.de; c.m.j.pieterse@uu.nl

Besides its key role in the onset of microbe-induced ISR, MYB72 has been demonstrated to play a critical role in the survival of Arabidopsis plants in soils where iron (Fe) availability is restricted (Palmer *et al.* 2013). In response to Fe starvation, *MYB72* is rapidly up-regulated in Arabidopsis roots as part of a set of coordinated responses, collectively referred to as the Strategy I Fe deficiency response, which boosts Fe mobilization and uptake from the soil (Connolly & Guerinot 2002; Buckhout *et al.* 2009; Zamioudis *et al.* 2014; Zamioudis *et al.* 2015; Verbon *et al.* 2017). The core of this Fe uptake response includes acidification of the rhizosphere via proton extrusion by H^+-ATPases (Santi & Schmid 2009), reduction of ferric Fe (Fe^{3+}) to ferrous Fe (Fe^{2+}) via the plasma membrane protein FRO2 (FERRIC REDUCTION OXIDASE2; Robinson *et al.* 1999) and the subsequent transport of ferrous Fe from the soil into root cells via the high-affinity ferrous Fe transporter IRT1 (FE-REGULATED TRANSPORTER1; Eide *et al.* 1996). Recently, it was demonstrated that MYB72 regulates the production and secretion of Fe-mobilizing phenolic metabolites by the plant root, thereby contributing to the Fe uptake machinery that is activated during Fe-limiting conditions (Zamioudis *et al.* 2014). A central player in the regulation of the Strategy I Fe deficiency response is the basic helix–loop–helix (bHLH) transcription factor FIT (FER-LIKE IRON DEFICIENCY TRANSCRIPTION FACTOR; Yuan *et al.* 2005; Bauer *et al.* 2007). In Arabidopsis, FIT interacts with other members of the bHLH family (bHLH38/39/100/101) to form heterodimers that regulate the expression of the Fe uptake genes *FRO2*, *IRT1* and *MYB72* (Yuan *et al.* 2008; Wang *et al.* 2013; Zamioudis *et al.* 2015).

Colonization of Arabidopsis roots by beneficial ISR-inducing rhizobacteria activates both *MYB72* and the Fe uptake genes *FRO2* and *IRT1* (Zamioudis *et al.* 2015), suggesting a mechanistic link between Fe homeostasis and the onset of ISR. Volatile compounds (VCs) released by ISR-inducing rhizobacteria emerged as important elicitors of the expression of *MYB72*, *FRO2* and *IRT1* in the roots of Arabidopsis, which was shown to be independent of Fe availability (Zhang *et al.* 2009; Zamioudis *et al.* 2014, 2015). Bacterial VCs have been recognized as info-chemicals mediating communication between bacteria and their host plants (Bailly & Weisskopf 2012). Bacterial VCs can be detected by neighbouring plants, leading to adjustments in plant growth and defensive status (Wenke *et al.* 2010). For example, exposure to VCs produced by certain rhizobacteria led to significant changes in root architecture and shoot biomass (Ryu *et al.* 2003; Blom *et al.* 2011; Meldau *et al.* 2013; Zamioudis *et al.* 2013; Delaplace *et al.* 2015) or to enhanced resistance against pathogen infection (Ryu *et al.* 2004; Chung *et al.* 2016). In most of the studies, complete plant seedlings were exposed to bacterial VCs. Hence, it is still unknown whether bacterial VCs are perceived by the roots, leading to a systemic signal that induces immunity in the leaves, or by the leaves. Furthermore, bacterial VCs can also directly inhibit fungal growth (Quintana-Rodriguez *et al.* 2015), making it difficult to discern between direct and plant-mediated effects of bacterial VCs on plant pathogens.

Whereas the effects of bacterial VCs on plant growth and immunity are relatively well documented, studies on the impact of VCs from root-associated fungi are much scarcer. For *Trichoderma* spp., a bouquet of over 40 fungal VCs (Jelen *et al.* 2014) were shown to be capable of triggering growth promotion, adjustments in root architecture and enhanced immunity in Arabidopsis (Hung *et al.* 2013; Garnica-Vergara *et al.* 2015; Kottb *et al.* 2015). In this study, we investigated the role of VCs released by the ISR-inducing *Trichoderma* strains *T. asperellum* T-34 (T-34) and *Trichoderma harzianum* T-78 (T-78) in the induction of *MYB72*, Fe deficiency responses and ISR in Arabidopsis. Moreover, we tested whether these *Trichoderma* VCs-mediated responses also occur in another plant species, tomato. *Trichoderma* VCs triggered *MYB72* expression and activated the Strategy I Fe deficiency response in Arabidopsis roots. We show that the induced root responses lead to priming of foliar tissues for enhanced JA-dependent defences and resistance against the fungal necrotrophic pathogen *Botrytis cinerea*. By using grafts of fungal VCs-exposed and non-exposed Arabidopsis seedlings, we were able to demonstrate that perception of *Trichoderma* VCs by the roots transduce a so far unknown ISR signal systemically to the leaves, which then become primed for JA-dependent defences. Moreover, we show that *Trichoderma* VCs also trigger Fe deficiency responses and immunity in tomato plants, suggesting that this response to microbial VCs is expressed in different plant species.

MATERIALS AND METHODS

Plant and fungal material and growth conditions

In this study, we used *A. thaliana* wild-type accession Col-0, the Arabidopsis reporter line *pMYB72:GFP-GUS* (Zamioudis *et al.* 2015) and tomato (*Solanum lycopersicum*) cv Moneymaker. Arabidopsis seeds were surface sterilized and sown on Murashige and Skoog (MS) agar-solidified medium supplemented with 0.5% sucrose and MES (2.5 mM), pH 6, in one of the compartments of two-compartment circular plates (120 mm diameter). After 2 d of stratification at 4 °C, the plates were positioned vertically and placed in a growth chamber (22 °C, 10 h: 14 h, light: dark; light intensity 100 μmol m^{-2} s^{-1}) for 12 d. Tomato seeds were surface sterilized and sown on MS agar-solidified medium supplemented with 0.5% sucrose and MES (2.5 mM), pH 6, in square plates (120 × 120 mm) containing a smaller (60 mm diameter) circular plate. Plates were positioned vertically and placed in a growth chamber (22 °C, 10 h: 14 h, light: dark; light intensity 100 μmol m^{-2} s^{-1}) for 10 d.

For pathogen resistance bioassays, Arabidopsis and tomato seedlings that were grown on plates for 15 and 13 d, respectively, were transferred to 60 and 400 mL pots, respectively, containing a sand:soil mixture (5:12, *v:v*) that had been autoclaved twice with a 24 h interval. Arabidopsis and tomato plants were cultivated in a growth chamber with an 8 h light (24 °C, light intensity 100 μmol m^{-2} s^{-1}) and 16 h dark (20 °C) cycle at 70% relative humidity. Plants were watered every other day and received half-strength Hoagland solution containing 10 μM sequestreen (CIBA-Geigy) once a week.

Trichoderma asperellum T-34 (Segarra *et al.* 2009) and *T. harzianum* T-78 (Martinez-Medina *et al.* 2009) were cultured

at 28 °C on potato dextrose agar plates for 5 d as described by Martinez-Medina *et al*. (2014). A 7 mm diameter plug of each *Trichoderma* strain obtained from actively growing margins of potato dextrose agar cultures was transferred into the plant-free compartment containing MS agar-solidified medium. Unless stated otherwise, the plates were sealed with one layer of gas-permeable Parafilm (Sigma) and placed in a vertical position in the growth chamber. In the two-compartment plates, seedlings and microbes were physically separated, but gas exchange was allowed between the compartments.

Fluorescence microscopy

Confocal laser-scanning microscopy was performed with a Zeiss LSM 700 microscope as described by Zamioudis *et al.* (2015). Green fluorescent protein (GFP) in the *pMYB72:GFP-GUS* reporter line was excited by using the 488 nm Argon laser and fluorescence was detected at 500–550 nm. As counterstain, roots were stained with 10 μg mL^{-1} propidium iodide solution for 2 min, and fluorescence was detected at 570–620 nm. GFP fluorescence in *pMYB72:GFP-GUS* was also examined on a Leica MZ16FA fluorescence stereo microscope equipped with GFP3 filter.

Root ferric-chelate reductase activity

Ferric-chelate reductase activity was visualized by transferring 15 and 13-day-old Arabidopsis and tomato seedlings, respectively, onto agar-solidified Hoagland medium (Hoagland & Arnon 1938) supplemented with 0.5 mM CaSO4, 0.5 mM Ferrozine and 0.5 mM Fe(III)EDTA and incubating them for 20 min (Schmidt *et al*. 2000). As positive control, plants grown under Fe starvation were used. Fe deficiency was induced by transferring seedlings grown in standard Hoagland agar-solidified medium onto plates containing Hoagland medium from which Fe was omitted and incubating them for 3 d.

Root morphology assessment

Root systems were photographed, and primary root length and lateral root length were measured by using Fiji software (Schindelin *et al.* 2012). The number of visible lateral roots per plant was also counted. Root apical and subapical root hair patterns were observed by using stereo microscope ASKANIA GSZ T2 (Mikroskop Technik Rathenow).

Chemical treatments

To study the ability of *Trichoderma* VCs to prime JA-regulated defences, shoots of seedlings that were grown in the two-compartment plates were sprayed with 10 μM methyl JA (MeJA) solution (Brunschwig Chemie), which was prepared from a 100 mM MeJA stock solution in 96% (*v*:*v*) ethanol. The MeJA concentration was chosen based on pilot bioassays in which different concentrations of MeJA (from 10 to 100 μM) were tested (data not shown).

Botrytis cinerea bioassays

Five-week-old Arabidopsis or tomato plants were inoculated with *B. cinerea* strain B05.10 (Van Kan *et al*. 1997) according to Van Wees *et al*. (2013). The inoculation was achieved by applying a 5 μL droplet of a suspension of 1×10^5 spores mL^{-1} to four leaves per plant. Plants were subsequently placed under a lid to increase relative humidity to 100% in order to stimulate the infection. Disease symptoms were scored 2 and 3 d after *B. cinerea* inoculation by visual inspection. Disease ratings were assigned on each leaf according to Van der Ent *et al.* (2008). Percentage of leaves in each class was calculated per plant. Shoot samples for quantifying *B. cinerea* DNA were harvested 2 d after inoculation.

Grafting

Grafts were performed according to Marsch-Martinez *et al.* (2013) with minor modifications. Uniform 8-day-old seedlings were mock treated (no *Trichoderma* spp. on the split plates) or treated for 3 d with VCs from T-34 or T-78 in the two-compartment plates (T-34 or T-78 was present in the plant-free compartment). Then, the seedlings were transferred to square plates (120 × 120 mm) containing a thin layer of 1% agarose, after which first the cotyledons were removed. Subsequently, hypocotyls were cut. The cut plant pieces were transferred to the recovery plates (MS agar-solidified medium supplemented with 0.5% sucrose, pH 6) where grafts were assembled by joining the scions and rootstocks. After grafting, plates were sealed and kept horizontally for 7 d. Successful graft formation was evaluated by the attachment of scion to rootstock and the resumption of root growth. The success rate of grafting was approximately 50%.

Real-time quantitative RT-PCR

For the determination of fungal biomass in infected plant material, the total DNA of leaves of Arabidopsis and tomato plants was extracted by using the DNeasy plant kit (Qiagen) according to the manufacturer's instructions. For gene expression analyses, total RNA of leaves and roots of Arabidopsis and tomato plants were extracted by using the GenElute™ Plant total RNA kit (Sigma) according to the manufacturer's instructions and treated with DNase (Thermo Scientific). First-strand cDNA was synthesized from 1 μg of purified total RNA by using the Revert Aid H Minus RT (Thermo Scientific) according to the manufacturer's instructions. Real-time quantitative RT-PCR reactions and relative quantification of specific DNA and mRNA levels were performed according to Vos *et al.* (2015) and by using the gene-specific primers described in Supporting Information Table S1. For gene expression analysis, the data were normalized by using the housekeeping genes *Actin7* (At5g09810) and *SlEF* (encoding an elongation factor, X14449) for Arabidopsis and tomato, respectively. Fungal DNA was determined by analysing *B. cinerea Tubulin* gene (XM_001560987.1) relative to the Arabidopsis *At1g13320* or tomato *SlEF* genes.

RESULTS

Trichoderma VCs trigger *MYB72* and the Fe deficiency response in Arabidopsis roots

Previously, VCs from beneficial rhizobacteria were shown to trigger a Fe deficiency response in Arabidopsis roots (Zhang *et al.* 2009; Zamioudis *et al.* 2015). Here, we first investigated whether VCs released by beneficial rhizofungi activate similar Fe uptake mechanisms. We tested the VCs that are released by the well-characterized ISR-inducing *Trichoderma* fungi *T. asperellum* T-34 (T-34) and *T. harzianum* T-78 (T-78) for their ability to stimulate the expression of the root-specific ISR regulatory gene *MYB72* (Van der Ent *et al.* 2008) and the Fe deficiency-marker genes *FRO2* and *IRT1* (Connolly *et al.* 2002, 2003). To this end, Arabidopsis seedlings were treated with VCs from T-34 or T-78 by using a split-plate system. After 2 and 3 d of treatment, root samples were collected for gene expression analysis. Figure 1a shows that VCs released by T-34 and T-78 significantly up-regulated *MYB72*, *FRO2* and *IRT1*, which was greater at 2 d than at 3 d after VCs treatment. *Trichoderma* VCs further induced the up-regulation of the genes *bHLH38* and *bHLH39* (Fig. 1b), which encode two bHLH transcription factors that can form heterodimers with the bHLH transcription factor FIT that regulates the induction of *FRO2* and *IRT1* under Fe limiting conditions (Yuan *et al.* 2008). The *bHLH38* gene was strongly up-regulated both at 2

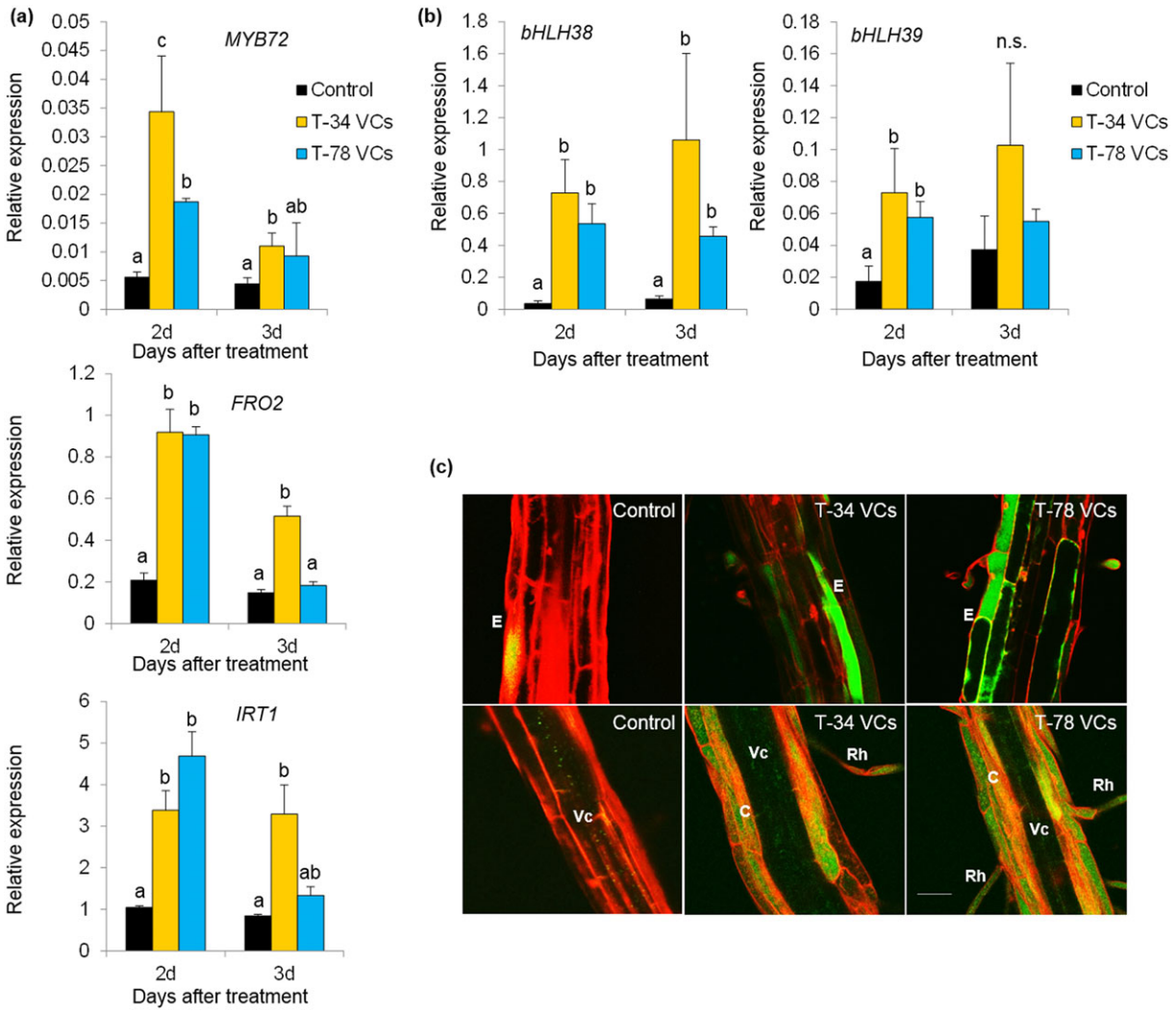

Figure 1. Volatile compounds (VCs) from *Trichoderma* T-34 and T-78 elicit Fe deficiency response-marker genes in Arabidopsis roots. Relative expression of *MYB72*, *FRO2* and *IRT1* (a) and *bHLH38* and *bHLH39* (b) in roots of Arabidopsis, mock-treated (control) or treated with VCs from *Trichoderma asperellum* T-34 (T-34 VCs) or *Trichoderma harzianum* T-78 (T-78 VCs). Seedlings were treated with *Trichoderma* VCs in split-plate assays for 2 or 3 d. Expression was normalized to that of *ACTIN7*. Values are means ± SE of five biological replicates. Each biological replicate consisted of pooled root tissue from four split plates, containing 12–15 Arabidopsis seedlings per plate. The different letters at each time point indicate statistically significant differences (Tukey HSD test; $P < 0.05$; n.s., not significant). (c) Representative confocal images of *pMYB72:GFP-GUS* roots that were mock-treated (control) or treated with VCs from T-34 or T-78 for 2 d. The upper and lower panels show different optical sections. E, epidermis; C, cortex; Rh, root hair; Vc, vascular cylinder. Cell walls were counterstained with propidium iodide (red signal). Scale bar = 50 μm. These results are representative of three independent experiments.

and 3 d after VCs treatment, whereas *bHLH39* was specifically induced at 2 d.

To further investigate the cellular expression pattern of *MYB72* after root perception of *Trichoderma* VCs, we used the transgenic line *pMYB72:GFP-GUS* expressing the GFP-GUS fusion protein under control of the *MYB72* promoter (Zamioudis *et al.* 2015). In mock-treated control roots, *MYB72* was expressed at a low basal level mainly in the vascular bundle and in discrete regions of the root epidermis (Fig. 1c). However, VCs from T-34 and T-78 induced a strong accumulation of GFP fluorophore, mainly in the epidermal and cortical root cells and in root hairs (Fig. 1c).

To examine the distribution of the VCs-induced Fe-reduction activity, we analysed the *in situ* activity of ferric-chelate reductase in VCs-treated seedlings. Figure 2a shows that ferric-chelate reductase activity was enhanced in VCs-treated seedlings to the same extent as in seedlings grown under Fe deprivation. Ferric-chelate reductase activity in VCs-treated seedlings was observed both in the maturation and in the subapical root zones. Collectively, these results indicate that VCs from *Trichoderma* fungi stimulate *MYB72* and molecular and physiological Fe uptake mechanisms in Arabidopsis roots.

Trichoderma VCs trigger similar morphological responses as Fe deficiency in Arabidopsis roots

Increased root branching and root hair formation are common responses of Strategy I plants to low Fe supply (Schmidt 1999; Graziano & Lamattina 2007; Jin *et al.* 2008). We reasoned that the activation of molecular Fe uptake responses by *Trichoderma* VCs might be co-regulated with the activation of the morphological adaptive responses to low Fe availability. To investigate this, we compared the architecture and growth of Arabidopsis roots in response to *Trichoderma* VCs or Fe deprivation. As expected, Fe limitation increased the number of lateral roots (Fig. 2b), without affecting primary root length (Supporting Information Fig. S1). Fe limitation also stimulated root hair formation in lateral roots (Fig. 2c) and in the primary root (Supporting Information Fig. S1). Likewise, T-34 and T-78 VCs stimulated root branching and root hair formation, while primary root growth was unaffected (Figs 2b and 2c and Supporting Information Fig. S1). The stimulation of root hair formation by T-78 VCs was stronger than that stimulated by T-34 VCs (Fig. 2c). Interestingly, the number and length of lateral roots of VCs-exposed plants were higher than in Fe-deprived plants (Fig. 2b). Together, these observations indicate that fungal VCs induce morphological adaptive responses in Arabidopsis roots that resemble those observed in plants exposed to low Fe availability.

Trichoderma VCs prime JA-dependent defences in Arabidopsis leaves and induce resistance against *Botrytis cinerea*

Given the critical role of *MYB72* in *Trichoderma*-induced ISR (Segarra *et al.* 2009) and the strong impact of *Trichoderma* VCs on *MYB72* regulation in Arabidopsis roots, we next aimed to

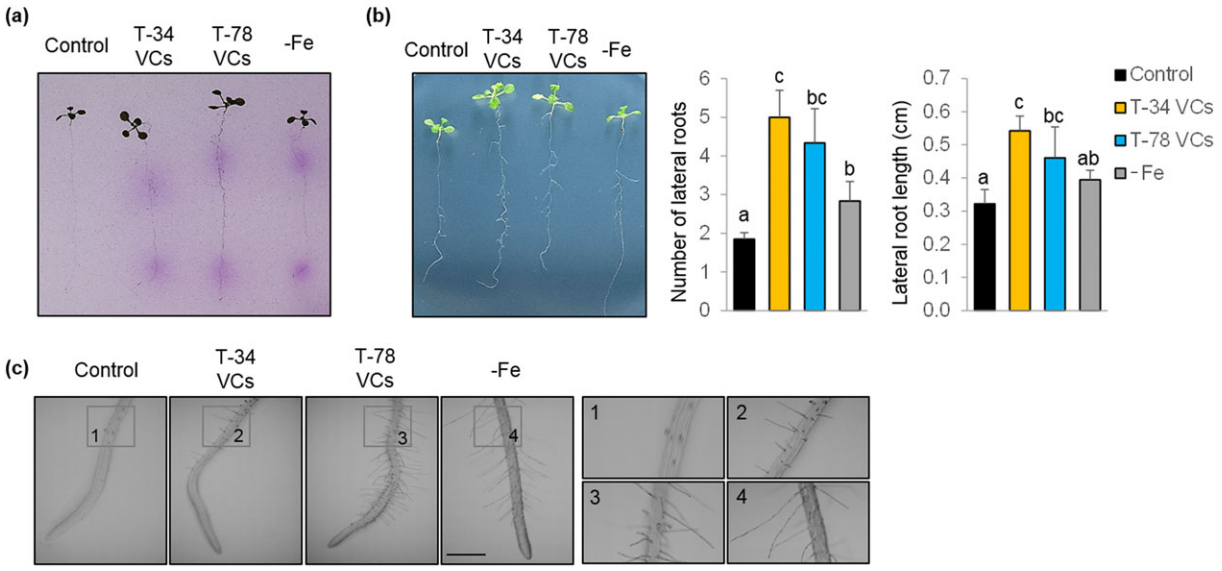

Figure 2. *Trichoderma* volatile compounds (VCs) induce ferric-chelate reductase activity and stimulate root branching and root hair formation. (a) Representative image of *in situ* localization of ferric-chelate reductase activity (purple colour) in Arabidopsis seedlings that were mock-treated (control), or exposed for 3 d to VCs from *Trichoderma asperellum* T-34 (T-34 VCs) or *Trichoderma harzianum* T-78 (T-78 VCs) in split-plate assays or grown under Fe-limited conditions (−Fe) for 3 d. (b) Representative photograph (left panel), number of lateral roots (middle panel) and length of lateral roots (right panel) of Arabidopsis seedlings that were mock-treated (control), or exposed for 3 d to T-34 VCs or T-78 VCs in split-plate assays or grown under Fe-limited conditions for 3 d. (c) Representative photograph of root hair development on lateral roots of Arabidopsis seedlings mock-treated (control), or treated for 3 d with T-34 VCs or T-78 VCs in the split-plate assays or grown under Fe-limited conditions (−Fe) for 3 d. In (b), values are means ± SE of 20 Arabidopsis seedlings. The different letters indicate statistically significant differences (Tukey HSD test; $P < 0.05$). Scale bar = 150 μm. These results are representative of three independent experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

study whether VCs released by T-34 and T-78 function as determinants for the elicitation of plant defences. To this end, seedlings were treated for 3 d with VCs of T-34 or T-78 in split-plate assays. Subsequently, shoots from mock-treated or VCs-treated seedlings were sprayed with 10 μM MeJA after which the expression of the JA-responsive marker genes *VSP2* (*VEGETATIVE STORAGE PROTEIN2*; Berger *et al.* 1995) and *PDF1.2* (*PLANT DEFENSIN1.2*; Penninckx *et al.* 1998) was analysed (Fig. 3a). As expected, MeJA application triggered the up-regulation of *VSP2* and *PDF1.2*, whereby transcriptional activation of *VSP2* was faster compared with *PDF1.2*. Treatment with T-34 or T-78 VCs was not associated with a direct transcriptional activation of the JA-responsive genes in Arabidopsis leaves, as shown by similar expression levels of *VSP2* and *PDF1.2* at time point 0 h for control and VCs-treated plants. However, after MeJA treatment, a faster and stronger up-regulation of *VSP2* and *PDF1.2* was observed in the leaves of plants that were previously treated with the fungal VCs compared with mock-treated plants, indicating that T-34 and T-78 VCs prime Arabidopsis seedlings for enhanced JA-responsive gene expression in the shoots.

Next, we investigated whether the priming for JA-responsive transcriptional induction by *Trichoderma* VCs is associated with enhanced resistance against pathogen attack. Mock-treated and VCs-treated seedlings were transplanted to pots, and 3 weeks later, the plants were inoculated with the

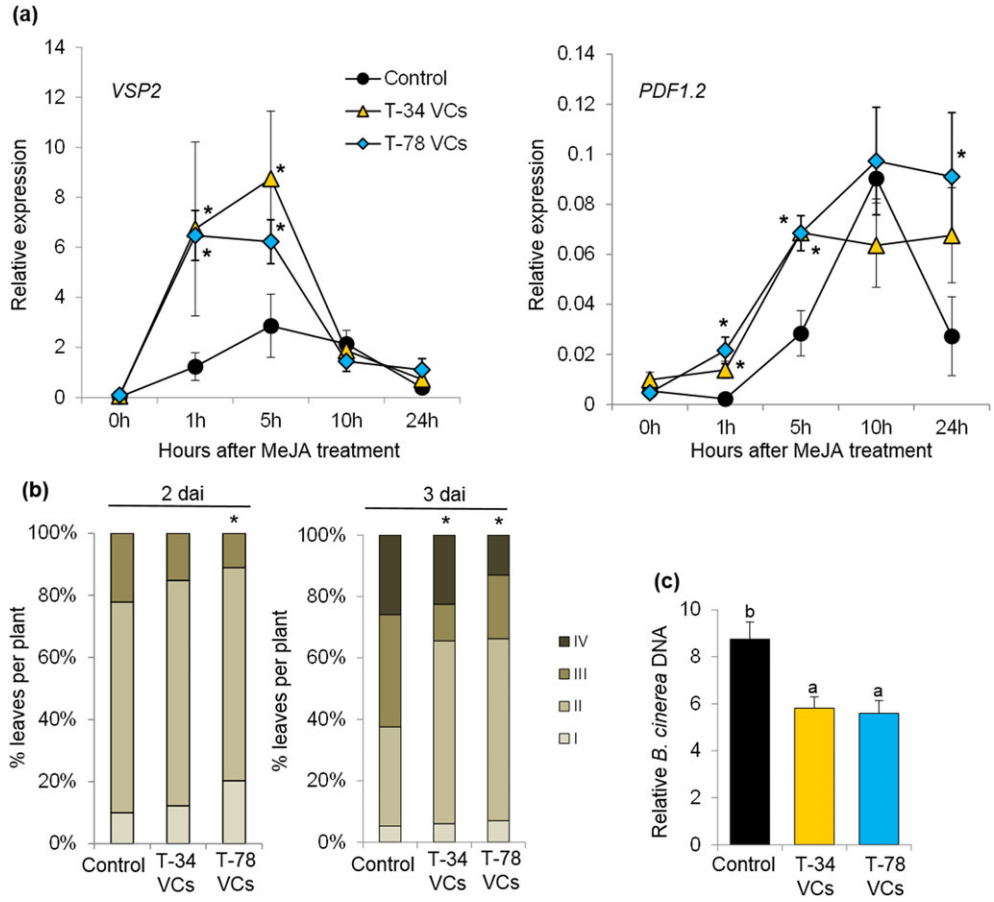

Figure 3. *Trichoderma* volatile compounds (VCs) prime Arabidopsis shoots for enhanced expression of jasmonic acid (JA)-responsive marker genes and induced resistance against *Botrytis cinerea*. (a) Relative expression of *VSP2* and *PDF1.2* in shoots of Arabidopsis seedlings that were mock-treated (control) or treated with VCs from *Trichoderma asperellum* T-34 (T-34 VCs) or *Trichoderma harzianum* T-78 (T-78 VCs) for 3 d, prior to exogenous application of MeJA. Relative expression at different time points after MeJA treatment was normalized to that of *ACTIN7*. Values are means ± SE of five biological replicates. Each biological replicate consisted of pooled shoot tissue from 12 to 15 Arabidopsis seedlings grown on one split-plate. For each time point, the asterisks indicate statistically significant differences compared with mock-treated seedlings (Dunnett test; $P < 0.05$). (b) Quantification of disease symptoms in Arabidopsis leaves after inoculation with *Botrytis cinerea*. Seedlings were mock-treated (control) or treated with VCs from T-34 or T-78 for 3 d in split-plate assays before transplanting them into pots, and inoculated 3 weeks later with *B. cinerea*. Disease severity was scored in four disease severity classes at 2 and 3 d after inoculation (dai): I, no visible symptoms; II, non-spreading lesion; III, spreading lesion without tissue maceration; IV, spreading lesion with tissue maceration and sporulation of the pathogen. Percentage of leaves in each class was calculated per plant. The asterisks indicate statistically significant differences compared with mock-treated control plants (χ^2 test; $n = 20$ plants). (c) Quantification of *B. cinerea* in Arabidopsis leaves. Relative amount of *B. cinerea* DNA was determined 2 d after inoculation by quantitative RT-PCR analysis of the *B. cinerea Tubulin* gene relative to the Arabidopsis *At1g13320* gene. Values are means ± SE of five biological replicates. The different letters indicate statistically significant differences between treatments (Tukey HSD test; $P < 0.05$). These results are representative of three independent experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

necrotrophic pathogen *B. cinerea*. As shown in Figs 3b and 3c, plants that were previously exposed to fungal VCs developed significantly less-severe disease symptoms (Fig. 3b) and contained reduced amounts of the pathogen, as determined by measurement of *B. cinerea Tubulin* DNA levels (Fig. 3c). Collectively, these results show that VCs from T-34 and T-78 prime Arabidopsis seedlings for enhanced JA-dependent defences that may contribute to the observed enhanced resistance against *B. cinerea*.

Micrografting reveals root-to-shoot signalling in *Trichoderma* VCs-induced resistance

Trichoderma-triggered ISR typically involves long-distance signalling that starts at the root-microbe interface. To study whether the enhanced protection elicited by *Trichoderma* VCs in leaves against *B. cinerea* is a phenomenon induced systemically, we made micrografts between mock-treated (control) scions and VCs-treated rootstocks (Fig. 4a). After a recovering period, the shoots of the grafts were sprayed with MeJA, after which the expression of *VSP2* and *PDF1.2* was analysed. Grafted seedlings composed of control scions and VCs-treated rootstocks displayed a stronger and longer-lasting up-regulation of *VSP2* and *PDF1.2* compared with control/control grafted seedlings (Fig. 4b). This suggests that the VCs perceived by the roots initiate a systemic signalling pathway that leads to priming for enhanced JA-responsive gene expression in the shoots. To test whether this VCs-mediated priming effect results in enhanced resistance against *B. cinerea*, grafted seedlings were transplanted to pots and inoculated with *B. cinerea* 3 weeks later. As shown in Figs 4c and 4d, control scions grafted onto rootstocks that were previously treated with fungal VCs were less susceptible to *B. cinerea* than control/control grafted plants. Protection of the plants was manifested by both a reduction in disease severity (Fig. 4c) and a decrease in pathogen proliferation in the leaves (Fig. 4d). These observations indicate that root perception of *Trichoderma* VCs induces long-distance signalling, which stimulates plant immunity in aboveground plant parts.

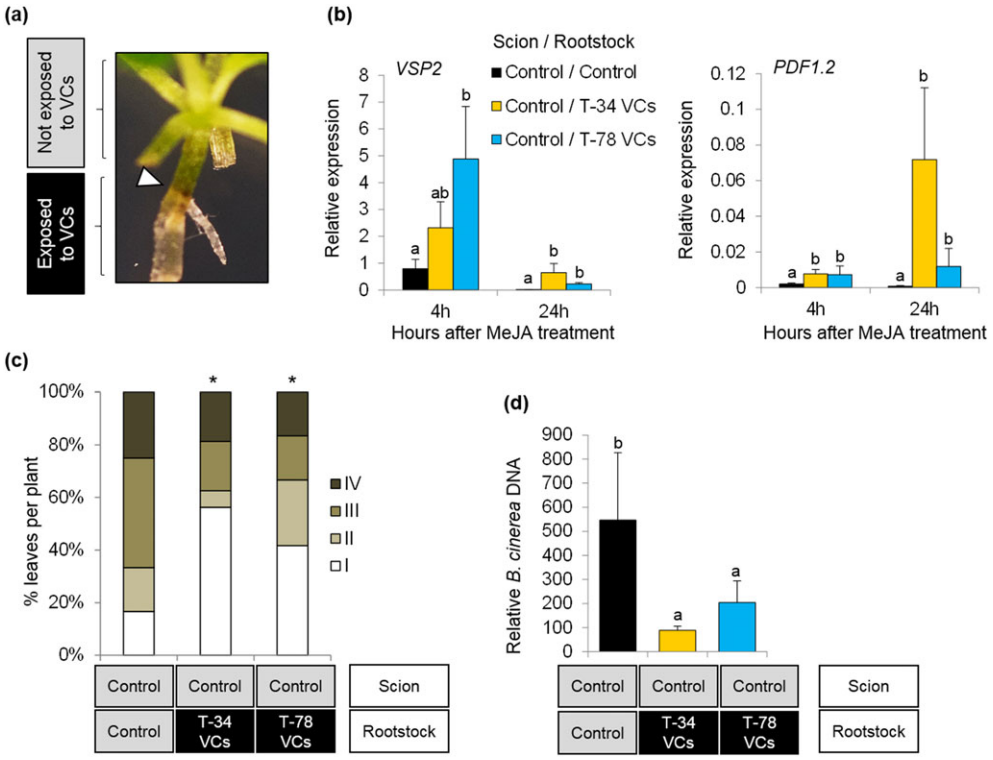

Figure 4. Root perception of *Trichoderma* volatile compounds (VCs) triggers systemic immunity in leaves of Arabidopsis grafts. (a) Micrografts of Arabidopsis seedlings were made between mock-treated (control) scions and rootstocks of seedlings that were treated for 3 d with VCs from *Trichoderma asperellum* T-34 (T-34 VCs) or *Trichoderma harzianum* T-78 (T-78 VCs). The white arrowhead points to the union site of the grafted seedling. (b) Relative expression of *VSP2* and *PDF1.2* in MeJA-treated leaves of control scions grafted onto mock-treated or VCs-treated rootstocks. Relative expression was normalized to that of *ACTIN7*. Values are means ± SE of four biological replicates. The different letters at each time point indicate statistically significant differences between grafts (Tukey HSD test; $P < 0.05$). (c) Quantification of *Botrytis cinerea* disease symptoms in control scions grafted onto mock-treated rootstocks or onto rootstocks treated with either T-34 VCs or T-78 VCs. Disease severity was scored in four disease severity classes at 2 d after inoculation: I, no visible symptoms; II, non-spreading lesion; III, spreading lesion without tissue maceration; IV, spreading lesion with tissue maceration and sporulation of the pathogen. Percentage of leaves in each class was calculated per plant. The asterisks indicate statistically significant differences compared with control/control grafted seedlings (χ^2 test; $n = 8$ plants). (d) Quantification of *B. cinerea* in leaves of Arabidopsis grafts. Relative amount of *B. cinerea* DNA was determined 2 d after inoculation by quantitative RT-PCR analysis of the *B. cinerea Tubulin* gene relative to the Arabidopsis *At1g13320* gene. Values are means ± SE of four biological replicates. The different letters indicate statistically significant differences (Tukey HSD test; $P < 0.05$). [Colour figure can be viewed at wileyonlinelibrary.com]

Trichoderma VCs stimulate Fe deficiency responses in tomato roots

To investigate whether we could reproduce the effects of fungal VCs also in other plant species than Arabidopsis, we investigated the impact of *Trichoderma* VCs on the Fe deficiency response in tomato (*S. lycopersicum*) seedlings. To this end, we first analysed whether VCs from T-34 and T-78 were capable of inducing the expression of the Fe deficiency response marker genes *LeFER* (encoding a bHLH transcription factor regulating expression of various Fe-uptake genes and controlling Fe homeostasis; Ling *et al.* 2002), *LeFRO* (encoding a Ferric-chelate reductase; Zamboni *et al.* 2012) and *LeIRT1* (encoding an Fe-regulated transporter; Zamboni *et al.* 2012). Tomato seedlings were treated with VCs from T-34 or T-78 in the split-plate system. After 2 d, root samples were collected for gene expression analysis. As shown in Fig. 5a, fungal VCs up-regulated the expression of *LeFER*, *LeFRO* and *LeIRT1*. In addition, ferric-chelate reductase activity was enhanced in roots of VCs-treated seedlings compared with mock-treated control seedlings (Fig. 5b). Ferric-chelate reductase activity in T-34 and T-78 VCs-treated seedlings was even higher than that in seedlings grown under Fe deprivation. VCs-induced ferric-chelate reductase activity was mainly associated with lateral roots (Fig. 5b).

We also investigated whether *Trichoderma* VCs induce morphological changes in tomato roots that are similar to those induced by low Fe availability. Treatment with T-34 or T-78 VCs stimulated lateral root formation to the same extent as the Fe deprivation treatment (Fig. 6a). In addition, T-34 and T-78 VCs stimulated the development of subapical root hairs, both in the primary root and in the lateral roots (Fig. 6b and Supporting Information Fig. S2) and subapical root swelling (Supporting Information Fig. S2). No significant differences were observed in the length of the primary or lateral roots of VCs-treated tomato plants compared with mock-treated controls (Supporting Information Fig. S2). Collectively, our results suggest that, like our observations in Arabidopsis, *Trichoderma* VCs elicited the molecular, physiological and morphological Fe uptake mechanisms in tomato roots.

Trichoderma VCs-induced resistance in tomato shoots

In analogy to Arabidopsis, we reasoned that *Trichoderma* VCs might systemically induce defences in the leaves of tomato plants. To investigate this, tomato seedlings were treated with VCs from T-34 and T-78 for 3 d in split-plate assays. Subsequently, shoots from mock-treated and VCs-treated seedlings

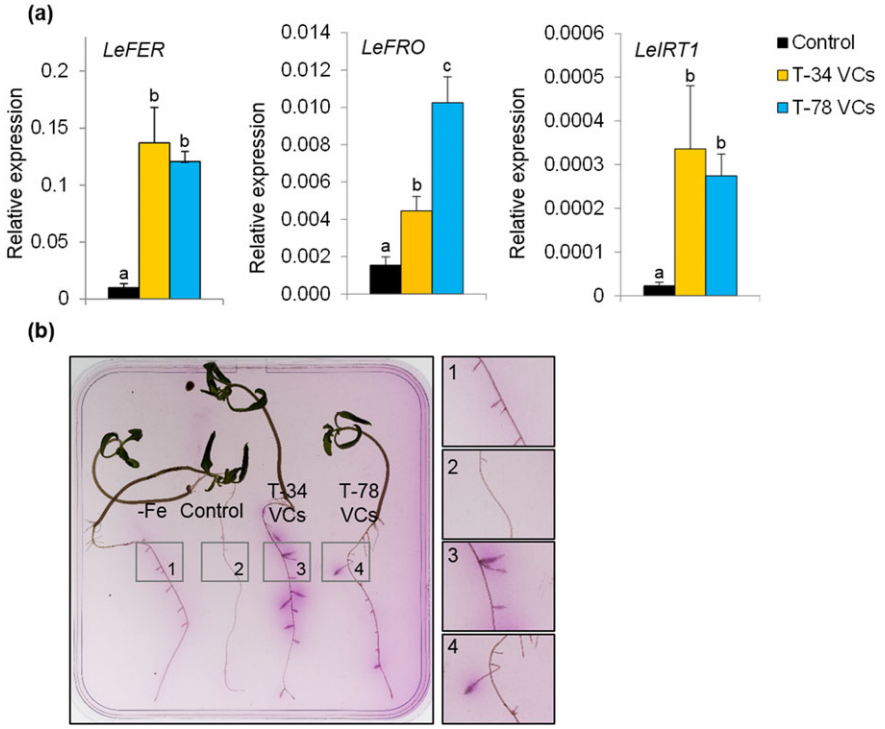

Figure 5. *Trichoderma* volatile compounds (VCs) trigger Fe deficiency response-marker genes and ferric-chelate reductase activity in tomato roots. (a) Relative expression of *LeFER*, *LeFRO* and *LeIRT1* in roots of tomato seedlings that were mock-treated (control) or treated with VCs from *Trichoderma asperellum* T-34 (T-34 VCs) or *Trichoderma harzianum* T-78 (T-78 VCs) for 2 d by using split-plate assays. Expression was normalized to that of the tomato reference gene *SlEF*. Values are means ± SE of five biological replicates. Each biological replicate consisted of pooled root tissue from five tomato seedlings grown in the same split-plate. The different letters indicate statistically significant differences between treatments (Tukey HSD test; $P < 0.05$). (b) Representative image of *in situ* localization of ferric-chelate reductase activity (purple colour) in tomato roots 3 d after treatment with mock (control), VCs from T-34 or T-78 or Fe limitation (−Fe). The magnified images show ferric-chelate reductase activity, associated with lateral roots. These results are representative of two independent experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

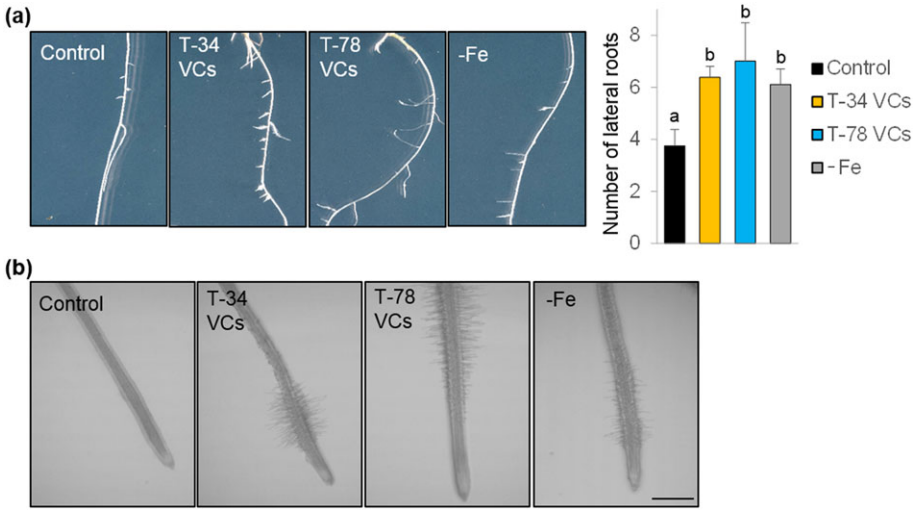

Figure 6. *Trichoderma* volatile compounds (VCs) stimulate root branching and root hair formation in tomato seedlings. (a) Representative photographs of root architecture and number of lateral roots of tomato seedlings that were mock-treated (control), or exposed for 3 d to VCs from *Trichoderma asperellum* T-34 (T-34 VCs) or *Trichoderma harzianum* T-78 (T-78 VCs) in split-plate assays or grown under Fe-limited conditions (−Fe) for 3 d. Values are means ± SE of 10 tomato seedlings. The different letters indicate statistically significant differences between treatments (Tukey HSD test; $P < 0.05$). (b) Representative photographs of root hair development on lateral roots of tomato seedlings that were mock-treated (control), or exposed for 3 d to T-34 VCs or T-78 VCs in split-plate assays or grown under Fe-limited conditions for 3 d. Scale bar = 150 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

were sprayed with MeJA, after which the expression of the JA-responsive genes *MC* (encoding a multicystatin; Uppalapati et al. 2005) and *GluB* (encoding a β-1,3-glucanase; Wu & Bradford 2003) was analysed in shoots, 4 and 24 h after MeJA treatment. As shown in Fig. 7a, seedlings that were previously treated with T-34 VCs displayed a stronger up-regulation of *MC* compared with mock-treated control seedlings, 24 h after MeJA treatment. However, no significant differences were observed 4 h after MeJA application. By contrast, T-78 VCs-treated seedlings showed a stronger up-regulation of *MC* compared with controls, 4 h after MeJA treatment, while 24 h after MeJA treatment, no differences in *MC* transcripts were detected (Fig. 7a). Seedlings treated with either T-34 or T-78 VCs both displayed a stronger up-regulation of *GluB* compared with mock-treated control seedlings, 24 h after MeJA treatment (Fig. 7a).

To gain further insight in the protective ability of *Trichoderma* VCs in tomato plants, tomato seedlings that were mock-treated or treated with T-34 or T-78 VCs in the split-plates were transplanted to pots. Three weeks after transplanting, the level of VCs-induced protection against *B. cinerea* was assessed. In analogy to Arabidopsis, pre-treatment with VCs from both *Trichoderma* fungi reduced disease severity (Fig. 7b) and pathogen proliferation in the leaves (Fig. 7c). Together, these results indicate that in analogy to Arabidopsis, tomato plants show priming for enhanced JA-responsive gene expression and increased protection against *B. cinerea* after exposure to *Trichoderma* VCs.

DISCUSSION

Root-associated microorganisms play important roles on plant performance by inducing growth promotion and triggering a broad-spectrum ISR (Pieterse *et al.* 2014). In Arabidopsis, the R2R3-type MYB transcription factor MYB72 has been shown to play an important role during both the onset of ISR mediated by different microbial mutualists (Van der Ent *et al.* 2008; Segarra *et al.* 2009) and in plant survival under low Fe availability (Van de Mortel *et al.* 2008; Palmer *et al.* 2013). Recently, VCs released by ISR-inducing rhizobacteria were implicated in the stimulation of *MYB72* and the Fe uptake genes *FRO2* and *IRT1* in Arabidopsis, linking early MYB72-dependent ISR signalling to processes related to the Fe deficiency response in plant roots (Zamioudis *et al.* 2015). In this study, we expanded on this knowledge by investigating the mode of action of VCs from ISR-inducing rhizofungi in the onset of MYB72-mediated ISR and Fe uptake-related responses in Arabidopsis and tomato plants.

VCs from *Trichoderma* fungi manipulate the Fe uptake machinery locally in the host root

Some *Trichoderma* species can have marked effects on plant nutrition by enhancing the bioavailability of nutrients in the rhizosphere and by influencing the root system architecture, thereby increasing the exploratory capacity of roots (Altomare *et al.* 1999; de Santiago *et al.* 2011; Contreras-Cornejo *et al.* 2015). *Trichoderma* siderophores and organic acids have been proposed to facilitate Fe acquisition by *Trichoderma*-roots (Li *et al.* 2015). Here, we demonstrated that *Trichoderma* can further improve plant Fe nutrition by manipulating the plant's own Fe acquisition machinery. We found that VCs released by the ISR-inducing *Trichoderma* fungi T-34 and T-78 are important elicitors of the Fe deficiency marker genes *FRO2*, *IRT1* and *MYB72* (Fig. 1), highlighting the ability of fungal VCs to

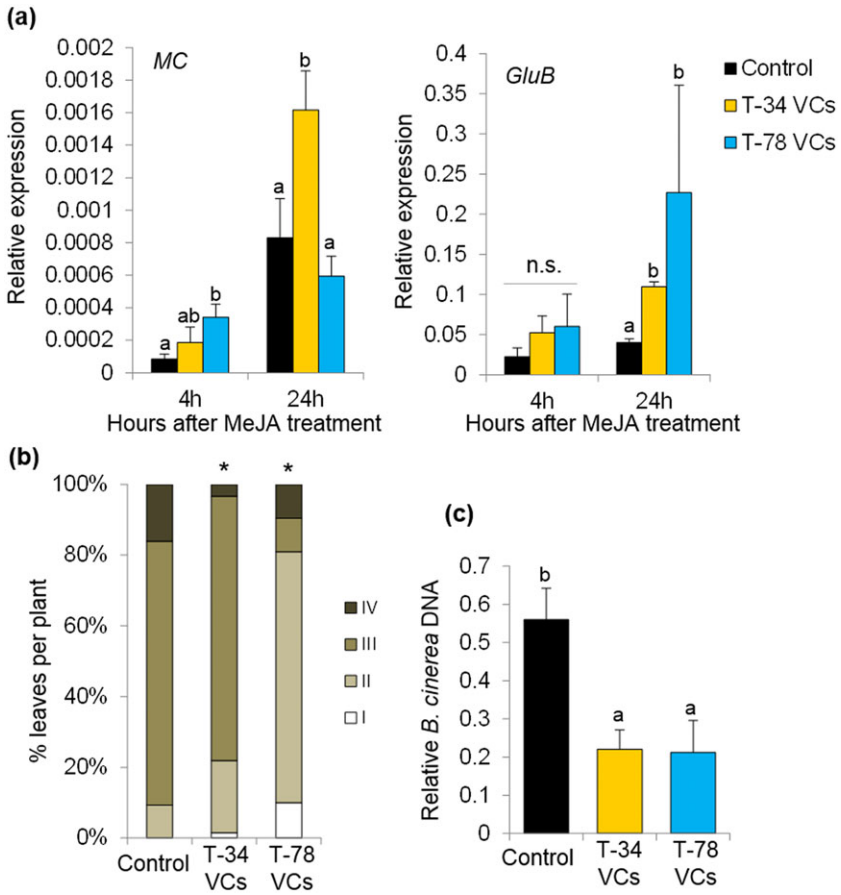

Figure 7. *Trichoderma* volatile compounds (VCs) prime tomato shoots for enhanced expression of jasmonic acid (JA)-responsive marker genes and enhanced resistance against *Botrytis cinerea*. (a) Relative expression of the JA-responsive genes *MC* and *GluB* in leaves of tomato seedlings mock-treated (control) or treated with VCs from *Trichoderma asperellum* T-34 (T-34 VCs) or *Trichoderma harzianum* T-78 (T-78 VCs) for 3 d, 4 and 24 h after exogenous application of MeJA. Relative expression was normalized to that of tomato reference gene *SlEF*. Values are means ± SE of four biological replicates. Each biological replicate consisted of pooled shoot tissue from five tomato seedlings grown in the same split-plate. The different letters at each time point indicate statistically significant differences (Tukey HSD test; $P < 0.05$). (b) Quantification of disease symptoms in tomato leaves after inoculation with *B. cinerea*. Seedlings were mock-treated (control) of treated with VCs from T-34 or T-78 for 3 d in split-plate assays before transplanting them into pots, and inoculated 3 weeks later with *B. cinerea*. Disease severity was scored 3 d after inoculation by using four disease severity classes: I, no visible disease symptoms; II, non-spreading lesion; III, spreading lesion without tissue maceration; IV, spreading lesion with tissue maceration and sporulation of the pathogen. Percentage of leaves in each class was calculated per plant. The asterisks indicate statistically significant differences compared with mock-treated control plants (χ^2 test; $n = 10$ plants). (c) Quantification of *B. cinerea* in tomato leaves. Relative amount of *B. cinerea* DNA was determined 3 d after inoculation by quantitative RT-PCR analysis of the *B. cinerea Tubulin* gene relative to the tomato reference gene *SlEF*. Values are means ± SE of five biological replicates. The different letters indicate statistically significant differences between treatments (Tukey HSD test; $P < 0.05$). These results are representative of two independent experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

stimulate Fe uptake mechanisms in the host roots. Induction of Fe uptake-related genes by microbial volatiles has been previously demonstrated for VCs of bacterial origin. VCs released by the plant growth-promoting rhizobacterium *Bacillus subtilis* GB03 and the ISR-inducing rhizobacterium *P. simiae* WCS417 similarly triggered the expression of Fe uptake-related genes in Arabidopsis roots, leading to elevated endogenous Fe levels in the plant (Zhang *et al.* 2009; Zamioudis *et al.* 2015). Accordingly, our results indicate that manipulation of Fe homeostatic mechanisms by microbial VCs is a feature conserved among different root-associated mutualists, ranging from bacteria to fungi.

It has been suggested that microbially produced CO_2, which can accumulate in sealed plates, can be partially involved in the growth response stimulated by microbial VCs (Kai & Piechulla 2009; Piechulla & Schnitzler 2016). Although plant growth promotion and Fe uptake responses elicited by microbial VCs are in our bioassays stronger than can be explained by elevated CO_2 alone (Zamioudis *et al.* 2015; Wintermans *et al.* 2016), we tested whether we could observe *MYB72* activation by *Trichoderma* VCs under non-sealed conditions, during which CO_2 produced by microbial activity equilibrates with the normal atmospheric CO_2 concentration by diffusing out of the Petri dish (Kai & Piechulla 2009). We found that the *Trichoderma* VCs similarly activated *MYB72* in Arabidopsis roots in the sealed and in the non-sealed setup (Supporting Information Fig. S3), indicating that other so-far unknown microbial VCs must play a role in the observed *Trichoderma*-stimulated root responses.

We found that VCs-mediated induction of *MYB72* was mainly restricted to the epidermal and cortical root cells and in root hairs (Fig. 1c). This localization coincides with the expression of the central Fe uptake regulatory gene *FIT* and the FIT-regulated genes *FRO2* and *IRT1* (Vert *et al.* 2002; Connolly *et al.* 2003; Colangelo & Guerinot 2004). Furthermore, *MYB72* has been identified as a component of the FIT regulatory network under low Fe availability conditions (Sivitz *et al.* 2012) and during plant responses to ISR-inducing rhizobacteria (Zamioudis *et al.* 2015). Stimulation of *MYB72* by fungal VCs was mainly restricted to the maturation zone (Fig. 1c). Induction of ferric-chelate reductase activity was expressed in the maturation zone of the roots and extended to a discrete zone of the subapical root area (Fig. 2a). This specific root zone has been previously associated with a strong ferric reduction activity and a strong accumulation of transcripts that are related to Fe acquisition (Santi & Schmidt 2008). Collectively, our results indicate that *Trichoderma* VCs activate Fe deficiency responses in root cell types that have previously been associated with Fe uptake.

Besides the activation of the Fe deficiency marker genes, *Trichoderma* VCs reshaped Arabidopsis root architecture by promoting root branching and root hair proliferation (Figs 2b and 2c). Increased lateral root emergence and root hair proliferation are typical adaptive responses to Fe deprivation (Schmidt 1999; Graziano & Lamattina 2007; Jin *et al.* 2008; Santi & Schmidt 2008). Both morphological responses to Fe deficiency have been associated with a higher exploratory capacity of the root and with the induction of the high affinity Fe uptake machinery (Schmidt *et al.* 2000; Jin *et al.* 2008). Collectively, our results show that fungal VCs enhance the capacity of the host plant to access and utilize Fe by modulating molecular, physiological and morphological adaptive responses to Fe-deficient conditions. The mechanisms by which microbial VCs activate Fe uptake responses in their host remain to be elucidated. We found that VCs from T-34 and T-78 increased shoot growth (Supporting Information Fig. S1). Increased shoot biomass is an Fe-demanding process that may result in the activation of the Fe uptake responses in the root. Accordingly, activation of the Fe deficiency response by VCs of plant growth-promoting rhizobacteria was shown to require photosynthesis-dependent signals from the shoot (Zamioudis *et al.* 2015). Alternatively, sucrose has been identified as a key regulator of the Fe deficiency responses in roots (Lin *et al.* 2016). Root colonization by beneficial symbionts constitutes an additional sucrose demand from the photosynthetic sources to the sink organs (Vargas *et al.* 2009; Doidy *et al.* 2012). Although further studies would be required, manipulation of sucrose transport or/and metabolism in the roots by root-associated symbionts might result in the activation of the Fe uptake responses in the host root.

Trichoderma VCs trigger systemic immunity in leaves by priming JA-dependent defences

Besides its critical role in Fe homeostasis, MYB72 was originally identify as an essential early component of *Pseudomonas* spp. and *Trichoderma* spp.-mediated ISR (Van der Ent *et al.* 2008; Segarra *et al.* 2009). The strong impact of fungal VCs on *MYB72* activation (Figs 1a and 1c) pointed to the possibility that microbial VCs may act as determinants for the elicitation of MYB72-dependent ISR. *Trichoderma*-ISR is generally not associated with a direct induction of cellular defences in shoots. Instead, ISR-expressing leaves are primed for enhanced activation of defences upon subsequent pathogen attack (Segarra *et al.* 2009; Martinez-Medina *et al.* 2013). In line with this, we found that treatment of seedlings with *Trichoderma* VCs was not associated with direct transcriptional activation of the JA-regulated genes *VSP2* and *PDF1.2* in leaves, but rather resulted in an accelerated and augmented induction of these genes in response to exogenous application of MeJA (Fig. 3a). Consequently, *Trichoderma* VCs conferred plant-mediated protection against the necrotrophic fungus *B. cinerea* (Figs 3b and 3c), evidencing the capability of fungal VCs to enhance the defensive capacity of leaves against pathogen attack.

Induction of shoot immunity by root-associated microbes is generally related to long-distance signalling that starts at the root-microbe interface (Pieterse *et al.* 2014). Most of the studies on the role of VCs in plant immunity involved the use of compartmental plates, wherein complete seedlings were exposed to microbial VCs (Ryu *et al.* 2004; Sharifi & Ryu 2016). In such experimental conditions, it is unclear whether VCs-induced immunity was triggered by root or shoot perception of microbial VCs. By using micrografting of VCs-exposed rootstocks and non-exposed scions of Arabidopsis seedlings, we demonstrated that perception of *Trichoderma* VCs by the root triggers systemic protection against *B. cinerea* in non-exposed leaves by priming for enhanced JA-inducible defences (Fig. 4). Although we cannot exclude the possibility that *Trichoderma* VCs might also stimulate immunity locally in the leaves, our results demonstrate that the root perception of *Trichoderma* VCs initiates a long-distance signalling that leads to enhanced immunity in systemic tissues. Evidence is emerging that suggests that the initiation of ISR results from a complex dialogue between the host plant and the microbial partner, during which both partners reciprocally communicate with chemical signals (Zamioudis & Pieterse 2012). For instance, several metabolites secreted by *Trichoderma* spp. with a recognized role in *Trichoderma*-root interaction have been shown to systemically activate plant defence mechanisms (Djonovic *et al.* 2006; Viterbo *et al.* 2007). Here, we demonstrated that airborne signals released by *Trichoderma* spp. are also strong determinants for the elicitation of ISR in the host plant.

VCs-mediated stimulation of Fe uptake responses and ISR is expressed in Arabidopsis and tomato

Dicotyledonous plants use Strategy I Fe deficiency responses to cope with Fe starvation (Brumbarova *et al.* 2015). To study whether *Trichoderma* VCs-mediated elicitation of Fe uptake mechanisms is a phenomenon that besides Arabidopsis is also expressed in other Strategy I species, we extend our studies to the crop species tomato. In tomato, Fe deprivation induces the activation of *LeFRO* and *LeIRT1*, which are the

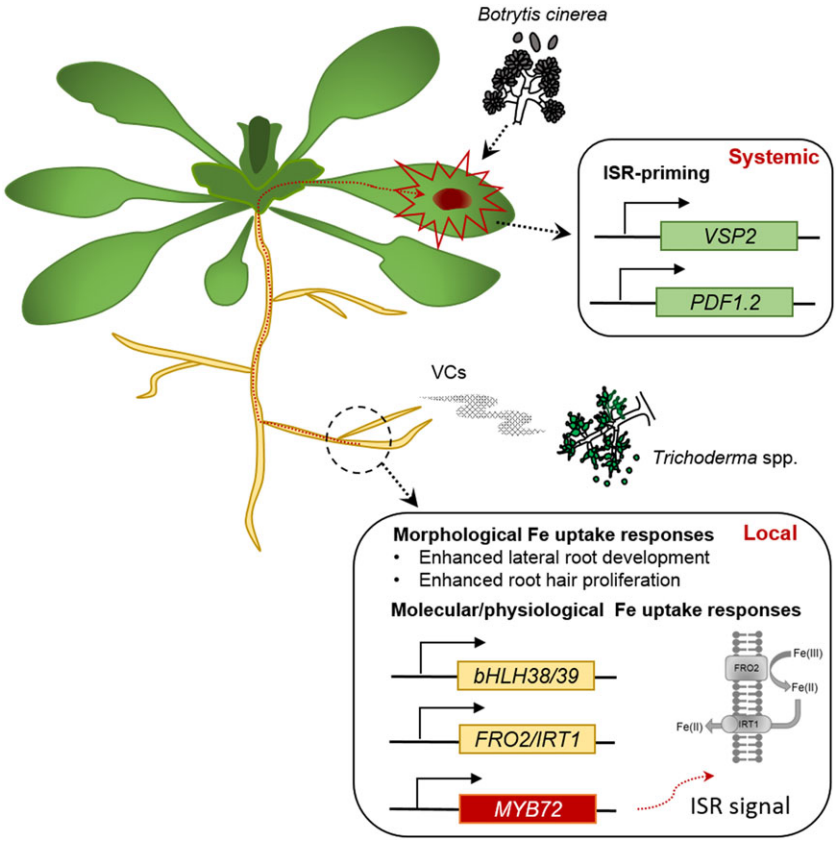

Figure 8. Model for local Fe uptake responses and systemic immunity triggered by *Trichoderma* volatile compounds (VCs). VCs from *Trichoderma* spp. are perceived by the host root, leading to the activation of *MYB72* and the Fe uptake-related molecular and morphological changes locally in the host root. Subsequently, a yet unknown induced systemic resistance (ISR) signal (red dots) is generated, which travels to systemic tissues, priming the leaves for enhanced jasmonic acid (JA)-regulated defences and triggering ISR against pathogen attack. [Colour figure can be viewed at wileyonlinelibrary.com]

homologues of Arabidopsis genes *FRO2* and *IRT1* (Bereczky *et al*. 2003; Connolly *et al.* 2003; Li *et al*. 2004). The bHLH transcription factor FER also accumulates upon Fe starvation and activates the transcription of several Fe deficiency-responsive genes, including *LeFRO* and *LeIRT1* (Ling *et al.* 2002; Brumbarova & Bauer 2005). In analogy to Arabidopsis, we found that VCs released by *Trichoderma* fungi activated the molecular and physiological responses to Fe deficiency in tomato (Figs 5a and 5b). Interestingly, VCs from the ISR-inducing rhizobacterium *P. simiae* WCS417 triggered a similar response (Supporting Information Fig. S4), highlighting the relevance of this phenomenon in the root interaction with the rhizosphere microbiota.

Tomato roots further responded to *Trichoderma* VCs through morphological changes in root architecture that are typically associated with low Fe supply, including the stimulation of root branching, the development of root hairs and root swelling (Fig. 6). Interestingly, a stronger VCs-mediated impact on root architecture was found in tomato, compared with Arabidopsis. Morphological root adaptations to Fe deprivation seem to be a species-specific phenomenon (Muller & Schmidt 2004; Santi & Schmidt 2008). Although part of the core of the Fe acquisition strategy, Fe stress-induced root hair formation in Arabidopsis indeed seems to be weaker compared with other species as tomato and cucumber (Santi & Schmidt 2008; Zamboni *et al.* 2012).

Finally, *Trichoderma*-VCs conferred plant-mediated protection against *B. cinerea* by priming tomato leaves for enhanced JA-defence responses (Fig. 7). The fact that two different plant species belonging to widely diverged families (*Cruciferae* and *Solanaceae*) show a similar response to microbial VCs indicates that airborne signals from soil microbes play a central role in the manipulation of the Fe acquisition machinery and the stimulation of host immunity during the interaction between plants and their microbial partners.

CONCLUSION

In this study, we demonstrate that airborne signals produced by beneficial root-colonizing *Trichoderma* fungi activate locally the Fe acquisition machinery in the roots of both Arabidopsis and tomato and that the activation of this process is associated with systemic priming of foliar tissues for enhanced JA-dependent defences and resistance against the necrotrophic fungus *B. cinerea* (Fig. 8). By using micrografting of Arabidopsis, we show that these VCs-mediated effects are initiated in the roots and thus must initiate a long-distance signalling process that ultimately results in ISR in the leaves (Fig. 8).

The mechanistic link between the transient activation of the MYB72-dependent Fe-deficiency response and the long-lasting elicitation of ISR is currently unknown. Interestingly, several studies have linked plant responses to changes in Fe homeostasis with changes in plant immunity (Kieu *et al.* 2012; Koen *et al.* 2014; Aznar *et al.* 2015). Our results further show that stimulation of the plant Fe uptake machinery and systemic immunity by soil mutualists is linked and is mediated by different root-associated microbes and plant taxa, highlighting the importance of these responses in plant interactions with rhizosphere microbiota.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. Impact of *Trichoderma* VCs on Arabidopsis root architecture and biomass. (a) Quantification of primary root length, (b) lateral root number relative to primary root length, (c) shoot biomass and representative images of shoots and (d) representative images of root hair development on the primary root of Arabidopsis seedlings. Seedlings were mock-treated (control), or exposed for 3 d to T-34 VCs or T-78 VCs in split-plate assays or grown under Fe-limited conditions for 3 d. In (a), (b) and (c), values are means ± SE of 20 Arabidopsis seedlings. Different letters indicate statistically significant differences (Tukey HSD test; $P < 0.05$); n.s., not-significant. Scale bar = 150 μm.

Figure S2. Impact of *Trichoderma* VCs on tomato root architecture. (a) Quantification of lateral and primary root length, (b) representative images of root hair development in the primary root and (c) representative photographs of root swelling in the subapical region of lateral roots of tomato seedlings. Seedlings were mock-treated (control), or exposed for 3 d to T-34 VCs or T-78 VCs in split-plate assays or grown under Fe-limited conditions for 3 d. In (a), values are means ± SE of 10 tomato seedlings. n.s., not-significant (Tukey HSD test; $P < 0.05$). Scale bar = 150 μm.

Figure S3. Impact of *Trichoderma* VCs on *MYB72* activation in sealed and in non-sealed setups. Representative confocal images of *pMYB72:GFP-GUS* roots that were mock-treated (control) or treated with VCs from T-34 or T-78 for 2 d in sealed (a) or non-sealed (b) split plate setups. Cell walls were counterstained with propidium iodide (red signal). Scale bar = 50 μm.

Figure S4. Volatiles from the ISR-inducing rhizobacterium *P. simiae* WCS417 elicit Fe deficiency response-marker genes in both Arabidopsis and tomato roots. (a) Relative expression of *MYB72*, *FRO2*, *IRT1*, *bHLH38* and *bHLH39* in roots of Arabidopsis and (b) relative expression of *LeFER*, *LeFRO2* and *LeIRT1* in roots of tomato seedlings. Arabidopsis and tomato seedlings were mock-treated (control) or exposed for 2 d to WCS417 VCs in the split-plate assays. Expression was normalized to that of Arabidopsis *ACTIN7* or tomato *SlEF*, respectively. Values are means ± SE of five biological replicates. Asterisks indicate statistically significant difference compared with mock-treated control seedlings (Student's *t*-test; $P < 0.05$).

Table S1. Primer sequences used in the quantitative RT-PCR analysis.