Molecular dialogue between arbuscular mycorrhizal fungi and the nonhost plant *Arabidopsis thaliana* switches from initial detection to antagonism

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Summary

- Approximately 29% of all vascular plant species are unable to establish an arbuscular mycorrhizal (AM) symbiosis. Despite this, AM fungi (*Rhizophagus* spp.) are enriched in the root microbiome of the nonhost *Arabidopsis thaliana*, and *Arabidopsis* roots become colonized when AM networks nurtured by host plants are available.
- Here, we investigated the nonhost–AM fungus interaction by analyzing transcriptional changes in *Rhizophagus*, *Arabidopsis* and the host plant *Medicago truncatula* while growing in the same mycorrhizal network.
- In early interaction stages, *Rhizophagus* activated the *Arabidopsis* strigolactone biosynthesis genes *CCD7* and *CCD8*, suggesting that detection of AM fungi is not completely impaired. However, in colonized *Arabidopsis* roots, fungal nutrient transporter genes *GintPT*, *GintAMT2*, *GintMST2* and *GintMST4*, essential for AM symbiosis, were not activated. RNA-seq transcriptome analysis pointed to activation of costly defenses in colonized *Arabidopsis* roots. Moreover, *Rhizophagus* colonization caused a 50% reduction in shoot biomass, but also led to enhanced systemic immunity against *Botrytis cinerea.*
- This suggests that early signaling between AM fungi and *Arabidopsis* is not completely impaired and that incompatibility appears at later interaction stages. Moreover, *Rhizophagus*-mediated defenses coincide with reduced *Arabidopsis* growth, but also with systemic disease resistance, highlighting the multifunctional role of AM fungi in host and nonhost interactions.

Introduction

The arbuscular mycorrhizal (AM) symbiosis is one of the most widespread mutualisms on Earth. It is established between soil fungi from the subphylum Glomeromycotina and the roots of *c.* 71% of all vascular plant species (Van der Heijden *et al.*, 2015; Brundrett & Tedersoo, 2018). In AM host plants, an extensive network of fungal hyphae increases the plant's exploratory capacity for water and mineral nutrients (Gutjahr & Parniske, 2013; Ferrol *et al.*, 2019). Besides improving plant nutrition, the symbiosis triggers significant changes in multiple host traits, such as root architecture, growth, development and stress tolerance (Martínez-Medina *et al.*, 2011; Jung *et al.*, 2012; Ruiz-Lozano *et al.*, 2012). In return, the plant supplies the fungus with lipids and sugars, at a cost of up to 20% of the carbon (C) fixed by photosynthesis (Smith & Read, 2008; Bravo *et al.*, 2017; Jiang *et al.*, 2017). It is known that AM fungi play a key role in ecosystems and they have been recognized as keystone taxa in microbial communities (Van der Heijden *et al.*, 2015; Keymer *et al.*, 2017; Luginbuehl *et al.*, 2017; Powell & Rillig, 2018; Banerjee *et al.*, 2018).

The intracellular accommodation of the AM fungus within the plant cell is a finely regulated process that results from a complex exchange of molecular information between the two partners (Gutjahr & Parniske, 2013; Fernández *et al.*, 2014; Pozo *et al.*, 2015). The development of the AM interaction starts with reciprocal exchange of diffusible signals before the symbiotic partners engage in physical contact (Bonfante & Genre, 2015). Host roots release strigolactones, which are signal molecules that are perceived by the fungal partner and subsequently induce extensive hyphal branching in the AM fungus (Giovannetti *et al.*, 1993; Buée *et al.*, 2000; López-Ráez *et al.*, 2011). Enhanced hyphal branching increases the probability of fungal contact with the host root (Besserer *et al.*, 2006). The host root, in turn, can perceive the signal molecules released into the rhizosphere by the

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fungal partner, known as 'myc factors' (Maillet *et al.*, 2011). Perception of myc factors triggers a specific Ca^{2+} spiking in root cells and activates a symbiotic program known as the 'sym pathway', which includes transcriptional, morphological and physiological changes in the host plant in order to accommodate the AM symbiosis (Gough & Cullimore, 2011).

After the exchange of diffusible signals between both partners, the fungus penetrates the root cortex. For a successful colonization, the fungus has to actively modulate defense signaling to reduce plant defense reactions in the host root to achieve a functional symbiosis. The AM fungus *Rhizophagus irregularis* (hereafter *Rhizophagus*) secretes effector proteins, such as SP7, which play a role in suppression of host defenses and accommodation of the fungus within plant roots (Kloppholz *et al.*, 2011). After root cortex colonization, fungal hyphae usually form arbuscules in which an exchange of C and nutrients between both partners occurs (Parniske, 2008; Bonfante & Genre, 2010; Harrison, 2012). Induced expression of fungal phosphate, ammonium and monosaccharide transporter genes is indicative of a mutually beneficial symbiotic interaction (Maldonado-Mendoza *et al.*, 2001; Helber *et al.*, 2011; Pérez-Tienda *et al.*, 2011). Genetic, genomic and phylogenetic analyses indicate that the ability to establish a functional symbiosis relies on a core set of symbiotic genes, the so-called 'symbiotic toolkit', which is highly conserved among plant families that host AM fungi (Delaux *et al.*, 2013). This symbiotic toolkit has been proposed to be required for the perception of AM fungi signals, root colonization, arbuscule development and control of the amount of root colonization (Lauressergues *et al.*, 2012).

Even though widespread, not all plant species form mycorrhizal associations and *c.* 29% of all vascular plant species are unable to establish an AM symbiosis (Brundrett & Tedersoo, 2018; Cosme *et al.*, 2018). These plants, denominated here as 'nonhost' plants, are abundant in families such as Brassicaceae, Chenopodiaceae, Polygonaceae, Amaranthaceae and Caryophyllaceae (Wang & Qiu, 2006), and include many important crops, such as rapeseed, sugar beet, broccoli, cauliflower, cabbage, spinach and chard. It has been proposed that ancestors of these plant families have independently lost the ability to form AM symbioses (Wang & Qiu, 2006). *Arabidopsis thaliana* (hereafter *Arabidopsis*) is the most studied model organism in plant biology and belongs to the group of nonhost plants (Lambers & Teste, 2013). The *Arabidopsis* genome lacks most genes of the symbiotic toolkit that are necessary for a functional AM symbiosis (Delaux *et al.*, 2014). Although considered as nonhosts, several studies demonstrate that typical nonhost plants can be colonized by AM fungi, especially when grown in the presence of other plant species that host a mycorrhizal network (Ocampo *et al.*, 1980; Francis & Read, 1995; Veiga *et al.*, 2013). This suggests that interactions between AM fungi and nonhost plants are more complex than generally recognized (Delaux *et al.*, 2014; Favre *et al.*, 2014; Bravo *et al.*, 2016).

Interestingly, some of the genes involved in the early dialogue between host plants and AM fungi are still present in the nonhost plant *Arabidopsis* (Delaux *et al.*, 2013, 2014). Among these are the strigolactone biosynthesis genes *CCD7* and *CCD8* (encoding two sequential carotenoid cleavage dioxygenase enzymes), which are upregulated during the early recognition process in compatible AM fungi–host interactions (Delaux *et al.*, 2014; López-Ráez *et al.*, 2015). Thus, it is reasonable to hypothesize that the presence of AM fungi might induce the expression of these genes and perhaps help to explain why there is occasional AM fungal colonization in nonhost plants. However, previous work revealed that such nonhost–AM fungus interactions often antagonize rather than promote plant growth (Veiga *et al.*, 2013), indicating that the molecular dialogue between AM fungi and host and nonhost plants can have contrasting outcomes. While host–AM fungus interactions are relatively well studied, in-depth analyses of interactions between nonhost plants and AM fungi are largely lacking. Therefore, we set out to investigate the molecular dialogue between the nonhost *Arabidopsis* and the AM fungus *Rhizophagus*, using an *in vitro* system for early stages of the interaction and the bicompartment microcosm system reported by Veiga *et al.* (2013) for later stages of the interaction.

Materials and Methods

Plant growth conditions and fungal inoculation in early interaction assays (*in vitro* experiments)

Arabidopsis thaliana Col-0 seeds were surface-sterilized as previously described (Van Wees *et al.*, 2013) and sown on ×1 Murashige & Skoog (MS) agar-solidified medium (Sigma-Aldrich) supplemented with 0.5% sucrose and 0.05% MES buffer. A density of 15 (fungal mycelium experiment) or five (germinating spore exudates experiment) seeds per square plate (120 × 120 mm) was used. After 2 d of stratification at 4°C, the Petri dishes were vertically positioned in a growth chamber under a short-day photoperiod (10 h : 14 h, light : dark, light intensity 100 μmol m^{-2} s^{-1}) at 21°C to initiate germination.

Fungal mycelium experiment For the *in vitro* fungal mycelium experiment, roots of 4-wk-old seedlings were exposed to the mycelium of the AM fungus *R. irregularis* DAOM 197198 (Tisserant *et al.*, 2012), the fungal root pathogen *Fusarium oxysporum* f.sp. *raphani* WCS600 (Pieterse *et al.*, 1996) or the beneficial root endophytic fungus *Trichoderma harzianum* T-78 (CECT 20714, Spanish collection of type cultures; Martínez-Medina *et al.*, 2013). To produce mycelium, *Rhizophagus* was grown in monoxenic cultures of *Agrobacterium rhizogenes* Ri T-DNA-transformed carrot roots (*Daucus carota* clone DC2) according to St-Arnaud *et al.* (1996). *In vitro Rhizophagus* cultures were established in bicompartmental Petri dishes to allow separation of the carrot root compartment from the compartment where only AM fungus hypha were allowed to grow. Cultures were started by placing a mycorrhizal carrot root segment in the root compartment containing M medium with 1% sucrose (Chabot *et al.*, 1992), after which they were incubated for 20 wk in the dark at 24°C until the hyphal compartment (M medium without sucrose) was profusely colonized by the AM fungus (Supporting Information Fig. S1a). *F. oxysporum* and *T. harzianum* mycelium was grown

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for 7 d on M medium with 1% sucrose in the dark at 28°C (Fig. S1b,c).

To expose *Arabidopsis* roots to each of the three fungi, pieces of medium containing fungal mycelium (*c.* one-quarter of a Petri dish) were placed in a new Petri dish, and uniform 4-wk-old *Arabidopsis* seedlings were transferred to the fungal cultures, 20 seedlings per plate, with the root placed on the surface of the culture and the shoot extending beyond the culture, in open air conditions (Fig. S1d–f). For the control treatment, 20 *Arabidopsis* seedlings were transferred to M medium without any fungal mycelium. After 4, 24 and 48 h of exposure of the roots to the fungal cultures, the roots were harvested for gene expression analysis. Roots from the 20 seedlings in a Petri dish were pooled to form one biological replicate. In all, six biological replicates were used for each fungal treatment.

Fungal structures and germinating spore exudates experiment After 4 wk of growth on MS plates, roots of *Arabidopsis* plants were exposed to a fungal structures suspension from *R. irregularis* DAOM 197198 (Tisserant *et al.*, 2012) by applying 300 µl (containing 250 spores) of fungal inoculant spore solution AGRONUTRITION (Quality D; Immeuble Biostep, Labege, France) on each root system. The fungal suspension consists of a mix of germinating spore exudates, spores and mycelium. The *Rhizophagus* spores were germinated as described by Mukherjee & Ané (2011). For the control treatment, 300 µl of sterile distilled water was applied to the *Arabidopsis* roots. After 2, 4, 24 and 48 h of exposure to the fungal structures suspension, roots were harvested for gene expression analysis. Roots from five seedlings grown in the same Petri dish were pooled to form one biological replicate. In all, six biological replicates were used.

Analysis of *Rhizophagus* abundance in *Arabidopsis* root microbiome

Twenty-day-old, *in vitro*-grown *A. thaliana* Col-0 seedlings were transferred from liquid Hoagland medium (2.5 mM inorganic phosphate (Pi)) (Rodríguez-Celma *et al.*, 2013) to 60 ml pots filled with soil from a field in the Reijerscamp nature reserve, the Netherlands (52°01′02.55″N, 5°77′99.83″E), where natural *Arabidopsis* populations grow as previously described (Berendsen *et al.*, 2018). Bulk soil pots were left unplanted. Rhizosphere and bulk soil samples (three root or soil samples per biological replicate; three biological replicates per treatment) were harvested at 3 d after transferring *Arabidopsis* plants from the *in vitro* system into the soil as previously described (Stringlis *et al.*, 2018b).

Shotgun metagenome sequencing and root microbiome analysis were performed as described previously (Stringlis *et al.*, 2018b). The raw metagenome read data are deposited in the Short Read Archive (http://www.ncbi.nlm.nih.gov/sra/; BioProject ID: PRJNA435676). Following sequencing, raw paired-end sequencing reads generated by NextSeq 500 were demultiplexed using BCL2FASTQ conversion software (v.2.17.1; Illumina, San Diego, CA, USA) according to the BaseSpace Illumina pipeline. FASTQC (v.0.11.5; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess length and quality of reads. Quality-filtered Illumina reads were taxonomically classified with Kaiju (Menzel *et al.*, 2016). Relative abundance of *Rhizophagus* spp., *Fusarium* spp. and *Trichoderma* spp. was calculated using the R language and environment (R, 2016; http://www.rproject.org) v.1.0.136, and the package PHYLOSEQ (McMurdie & Holmes, 2013).

Plant growth conditions and fungal inoculation in late interaction assays (*in vivo* experiments)

Arabidopsis thaliana Col-0 and *A. thaliana* T-DNA insertion mutant *max1* plants (Stirnberg *et al.*, 2002) were used as nonhost plants. *M. truncatula* A17 (hereafter *Medicago*) was used as host plant. Seeds from all these plants were surface-sterilized. *Arabidopsis* seedlings were pregrown in quartz sand for 3 wk in a growth chamber under short-day photoperiod (10 h : 14 h, light : dark, light intensity 100 µmol m^{-2} s^{-1}) at 21°C and 70% relative humidity before transfer to the microcosm with the *Medicago*-supported *Rhizophagus* AM fungal network.

The bicompartmental microcosm system used was similar to the one previously described by Veiga *et al.* (2013) with minor modifications. In brief, the system consisted of a microcosm with two equal compartments with a volume of 1 l each. The compartments were separated by a 30 µm nylon mesh to keep the *Arabidopsis* and *Medicago* root systems separated, while allowing host-supported *Rhizophagus* mycelium to colonize both compartments. Both compartments were filled with a river sand-potting soil mixture (5 : 12, v/v) that had been autoclaved twice for 20 min with a 24 h interval, supplemented with *Rhizophagus* inoculum or a mock treatment. The inoculum of *R. irregularis* BEG 21 was propagated on *Plantago lanceolata*, as described by Veiga *et al.* (2013). *R. irregularis* was previously referred to as *Glomus intraradices* and currently as *Rhizoglomus irregulare* (Sieverding *et al.*, 2015). The mycorrhizal inoculation was achieved by mixing 10% (w/w) of inoculum through the soil–sand mixture before adding it to the microcosm compartments. For the nonmycorrhizal compartments, a similar portion of autoclaved inoculum was mixed through the soil–sand mixture. In addition, through the soil of all compartments of the microcosm systems, 10 ml of a *Rhizophagus*-free filtrate (< 20 µm) of AM inoculum was mixed to homogenize the microbial populations of the mycorrhizal and nonmycorrhizal compartments. For this, 100 g of the AM fungus soil inoculum was suspended in 600 ml water and subsequently filtered as described by Veiga *et al.* (2013).

According to the treatment, four *Medicago* seeds were sown in one compartment of the microcosm and the adjacent compartment was left unsown. After 2 wk, 12 *A. thaliana* Col-0 or *max1* seedlings (3 wk old) were transferred to the other compartment of the block. Fig. S2 provides an illustration of the microcosm setup. The microcosms were then placed in a completely randomized design in the growth chamber under short-day photoperiod (10 h : 14 h, light : dark, light intensity 100 µmol m^{-2} s^{-1}) at 21°C and 70% relative humidity. Plants were watered three times a week, alternating with tap water and half-strength Hoagland solution (Hoagland & Arnon, 1938) containing only

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25% of the standard KH_2PO_4 concentration (final concentration 0.6 mM) in order to reduce Pi availability. Eight weeks after transplanting the *Arabidopsis* seedlings into the microcosm, *Arabidopsis* and *Medicago* plants were harvested, after which shoot weight was measured and root systems were thoroughly washed with tap water to collect them for assessing root colonization by AM fungi and for histological and gene expression analysis as described by López-Ráez *et al.* (2010). For RNA-seq data analysis, *Arabidopsis* and *Medicago* root systems were harvested, immediately frozen in liquid nitrogen (N) and stored at −80°C until processing.

Plant nutrient analysis

For the nutrient content analysis, 0.1 g of shoot DW (obtained after 7 d at 65°C) from 11-wk-old *A. thaliana* Col-0 and 10-wk-old *M. truncatula* A17 plants was digested by a Milestone Ethos I microwave digestion instrument (Milestone, Milano, Italy). The nutrient content was analyzed using inducively coupled plasma (ICP) spectroscopy (Iris intrepid II XD2 Thermo; Thermo Scientific, Waltham, MA, USA). In addition, the C and N content was determined using a Flash 1112 series EA C/N analyzer (Thermo Scientific). This analysis was performed at the CEBAS-CSIC ionomic service (Murcia, Spain).

Microscopic determination of mycorrhizal root colonization

For the determination of mycorrhizal root colonization, roots obtained from the *in vivo* experiments were stained with trypan blue solution (Phillips & Hayman, 1970) and examined using a Zeiss AxiosKop2 microscope and bright field conditions. The percentage of total root length colonized by the fungus was determined by the gridline intersection method using 200 intersections per sample (Giovannetti & Mosse, 1980).

Confocal laser-scanning microscopy of *Arabidopsis* and *Medicago* roots was performed using a Zeiss LSM 700 microscope. For fungal staining, random pieces of roots *c.* 1 cm in length were incubated for 30 min in 0.05 mg ml^{-1} wheat germ agglutinin (WGA; Alexa Fluor 488, ThermoFisher), after which they were washed three times for 5 min each, with phosphate-buffered saline (×1 PBS) according to Pérez-Tienda *et al.* (2011). Roots were counterstained in 10 μg ml^{-1} propidium iodide (PI) solution for 2 min. Chromophores were excited using the 488 nm argon laser and fluorescence was detected at 495–519 nm (WGA) and 570–620 nm (PI).

RNA sequencing

Total RNA was extracted from *A. thaliana* Col-0 and *M. truncatula* A17 roots as previously described (Stringlis *et al.*, 2018a). RNA-seq library preparation and sequencing were performed by KeyGene (Wageningen, the Netherlands). Sequencing libraries were prepared using the Illumina TruSeq RNA Sample Prep Kit (Illumina), and sequenced on the Illumina HiSeq2500 platform (Illumina) with read lengths of 125 bp. In total, 12 samples were loaded in a HiSeq2500 flow cell. For each of the four treatments, three biological replicates were sequenced, each with *c.* 30 million reads per sample with a read length of 125 bp, single or paired end for *Arabidopsis* and *Medicago*, respectively.

For read alignment, summarization and normalization, we followed the RNA-seq data analysis pipeline as previously described (Van Verk *et al.*, 2013; Coolen *et al.*, 2016; Hickman *et al.*, 2017). Reads were aligned to the *Arabidopsis* genome (TAIR version 10) and the *Medicago* genome (EnsemblGenomes) using TopHat v.2.0.452 (Center for Computational Biology, Baltimore, MD, USA) with the following parameter settings: 'transcriptome-mismatches 3', 'N 3', 'bowtie1', 'no-novel-juncs', 'genome-read-mismatches 3', 'p 6', 'read-mismatches 3', 'G', 'min-intron-length 40' and 'max-intron-length 2000'. Aligned reads were summarized over annotated gene models using HTSeq-count v.0.5.3p953 with settings '-stranded no' and '-i gene_id'. Sample counts were depth-adjusted using the median-count-ratio method available in the DESeq2 (Love *et al.*, 2014) (R v.3.3.1).

Genes that were significantly differentially expressed after *Rhizophagus* root colonization compared with noncolonized control roots were identified using DESeq2 (R v.3.1.1). Genes with $P \leq 0.05$ were called differentially expressed genes (DEGs). All statistics associated with testing for differential gene expression were performed with R (http://www.rproject.org).

Gene ontology (GO) enrichment analysis on gene clusters was performed using Plaza software (http://bioinformatics.psb.ugent.be/plaza/) (Proost *et al.*, 2015). Overrepresentation for the GO categories 'biological process' were identified by computing a *P*-value using the hypergeometric distribution and false discovery rate for multiple testing ($P \leq 0.05$).

The raw RNA-seq read data are deposited in the Short Read Archive (http://www.ncbi.nlm.nih.gov/sra/) and are accessible through BioProject ID number PRJNA 526801.

Gene expression analysis by real-time quantitative RT-PCR

Total RNA isolation from roots, cDNA synthesis and real-time quantitative reverse transcription polymerase chain reaction were performed as described previously (Stringlis *et al.*, 2018a) and using the gene-specific primers described in Table S1. Relative quantification of specific mRNA levels was performed using the comparative method of Livak & Schmittgen (2001). Expression values were normalized using the housekeeping genes *MtEF1α* and *At1g13320* (Czechowski *et al.*, 2005) for *Medicago* and *Arabidopsis*, respectively.

Plant immunity bioassays

Pots of 0.5 l volume were separated into two equal compartments by a 30 μm nylon mesh to keep the *A. thaliana* Col-0 and *M. truncatula* A17 root systems separated. Both compartments were filled with river sand-potting soil mixture as described earlier. *Medicago* compartments were supplemented with *R. irregularis* BEG 21 inoculum or with a mock treatment as described earlier. Single 1-wk-old *Medicago* seedlings, pregerminated in sterile vermiculite under short-day photoperiod (10 h : 14 h, light : dark, light intensity 100 μmol m^{-2} s^{-1}) were

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transplanted into the *Medicago* pot compartment. After 3 wk, single 3-wk-old *Arabidopsis* seedlings (pregerminated, as described earlier) were transferred into the *Arabidopsis* pot compartment. The bicompartmental pots were then placed in a completely randomized design in the growth chamber under a short-day photoperiod, at 21°C and 70% relative humidity. Plants were watered as described earlier. Six weeks after transplanting the *Arabidopsis* seedlings, eight leaves per *Arabidopsis* plant were inoculated with the foliar necrotrophic pathogen *Botrytis cinerea* B05.10 (Van Kan *et al.*, 1997) as described previously (Van Wees *et al.*, 2013). To this end, 5 µl droplets of a *B. cinerea* spore suspension (1×10^5 spores ml^{-1}) were applied to the leaves. Plants were placed at 100% relative humidity for 72 h to stimulate the infection. After 3 d, the disease severity was measured in all the plants infected by *B. cinerea*.

Statistical analysis

Data for shoot biomass and gene expression levels were subjected to one-way analysis of variance (ANOVA) using the software SPSS STATISTICS v.20 (IBM, Armonk, NY, USA) for Windows. Student's *t*-test, Dunnett's test and chi-squared test were applied with 5% significance level.

Results

Rhizophagus triggers strigolactone biosynthesis gene expression in *Arabidopsis* roots

In a first experiment, we explored whether *Arabidopsis* has the ability to detect the presence of *Rhizophagus* in the rhizosphere and whether the presence of *Rhizophagus* triggers early responses in *Arabidopsis* roots. In host plants, early recognition of signals from AM fungi elicits an increase in the expression of strigolactone biosynthesis genes, resulting in the increased production and exudation of strigolactones (Akiyama *et al.*, 2005; Kohlen *et al.*, 2012; López-Ráez *et al.*, 2015). In order to determine whether a similar response occurs in the roots of *Arabidopsis*, the expression of the *Arabidopsis* strigolactone biosynthesis genes *CCD7* and *CCD8* was analyzed in roots after placing the roots into contact with an *in vitro*-grown culture of *Rhizophagus* (Fig. S1). A significant increase in *CCD7* and *CCD8* transcript abundance was observed in roots of *Arabidopsis* within 24 h after transfer of the seedlings onto the mycorrhizal mycelium (Fig. 1a). The upregulation of *CCD7* and *CCD8* was still evident at 48 h after treatment. To eliminate the possibility that *CCD7* and *CCD8* were activated by nutrient deprivation in the fungal growth medium, we also tested whether direct application of a suspension of *Rhizophagus* fungal structures, consisting of germinating spore exudates, spores and mycelium, would induce *CCD7* and *CCD8* in *Arabidopsis* roots. After 24 h of exposure, we observed a significant induction of both *CCD7* and *CCD8* (Fig. 1b), suggesting that *Rhizophagus* activates these strigolactone biosynthesis genes in *Arabidopsis* roots.

To investigate the specificity of this plant response to the AM fungus, we analyzed *CCD7* and *CCD8* expression in *Arabidopsis* roots after treatment with mycelium from the pathogenic fungus *F. oxysporum* f.sp. *raphani* (hereafter *Fusarium*; Fig. S1) and the beneficial endophytic fungus *T. harzianum* (hereafter *Trichoderma*; Fig. S1). In contrast to the AM mycelium, the mycelium of *Fusarium* and *Trichoderma* did not significantly affect *CCD7* and *CCD8* transcript abundance compared with control plants (Fig. 1a). These results suggest that the induction of the strigolactone biosynthesis genes *CCD7* and *CCD8* in *Arabidopsis* roots interacting with *Rhizophagus* is part of a specific plant response triggered by the AM fungus, which resembles what is generally observed during the presymbiotic stages of host–AM fungi interactions.

Rhizophagus does not elicit early defense-related genes in *Arabidopsis* roots

During early stages of host–AM fungus interactions, plant defenses are modulated to allow the establishment and development of a functional AM symbiosis (García-Garrido & Ocampo, 2002). As the *Arabidopsis*–*Rhizophagus* interaction is described as a noncompatible association (Lambers & Teste, 2013; Veiga *et al.*, 2013), we hypothesized that *Rhizophagus* would activate costly defenses in the *Arabidopsis* roots during the early stages of the interaction. To test this hypothesis, we analyzed the expression of the well-characterized defense-related genes *MYB51* (encoding transcription factor MYB51, which regulates indolic glucosinolate biosynthetic pathway genes; Kranz *et al.*, 1998), *CYP71A12* (encoding cytochrome P450 71A12, which is involved in the biosynthesis of antimicrobial camalexin; Lin *et al.*, 1999), *PRB1* (encoding the basic pathogenesis-related protein PRB1, which is involved in defense responses against necrotrophic pathogens in roots; Santamaria *et al.*, 2001) and *ERF4* (encoding the transcription factor ERF4, which is involved in modulating ethylene responses; Yang *et al.*, 2005) in *Arabidopsis* roots after exposure to *in vitro*-grown *Rhizophagus* mycelium. Exposure of *Arabidopsis* roots to *Rhizophagus* mycelium did not significantly affect the transcript abundance of *MYB51*, *CYP71A12*, *PRB1* and *ERF4* (Fig. 1c). By contrast, exposure of *Arabidopsis* roots to mycelium of the pathogen *Fusarium* or the beneficial endophyte *Trichoderma* significantly induced the expression of these defense-related genes (Fig. 1c). These results indicate that, in contrast to the mycelia of *Fusarium* and *Trichoderma*, exposure of *Arabidopsis* roots to *Rhizophagus* mycelium does not immediately induce plant defense responses.

Rhizophagus is enriched in *Arabidopsis* rhizosphere

The potential of *Rhizophagus* to trigger strigolactone biosynthesis gene expression in *Arabidopsis* roots suggests that, similar to AM hosts, *Arabidopsis* may be able to attract *Rhizophagus* to its rhizosphere. In order to investigate this, we analyzed the fungal communities in the rhizosphere microbiome of *Arabidopsis* plants growing in natural soil and compared it with that of unplanted bulk soil. Interestingly, we observed a significant enrichment in the relative abundance of AM *Rhizophagus* spp. in the rhizosphere of *Arabidopsis* relative to the bulk soil, whereas other root-associated fungal species, such as *Fusarium* and *Trichoderma*, were not enriched (Fig. 2). This suggests that *Rhizophagus* spp.

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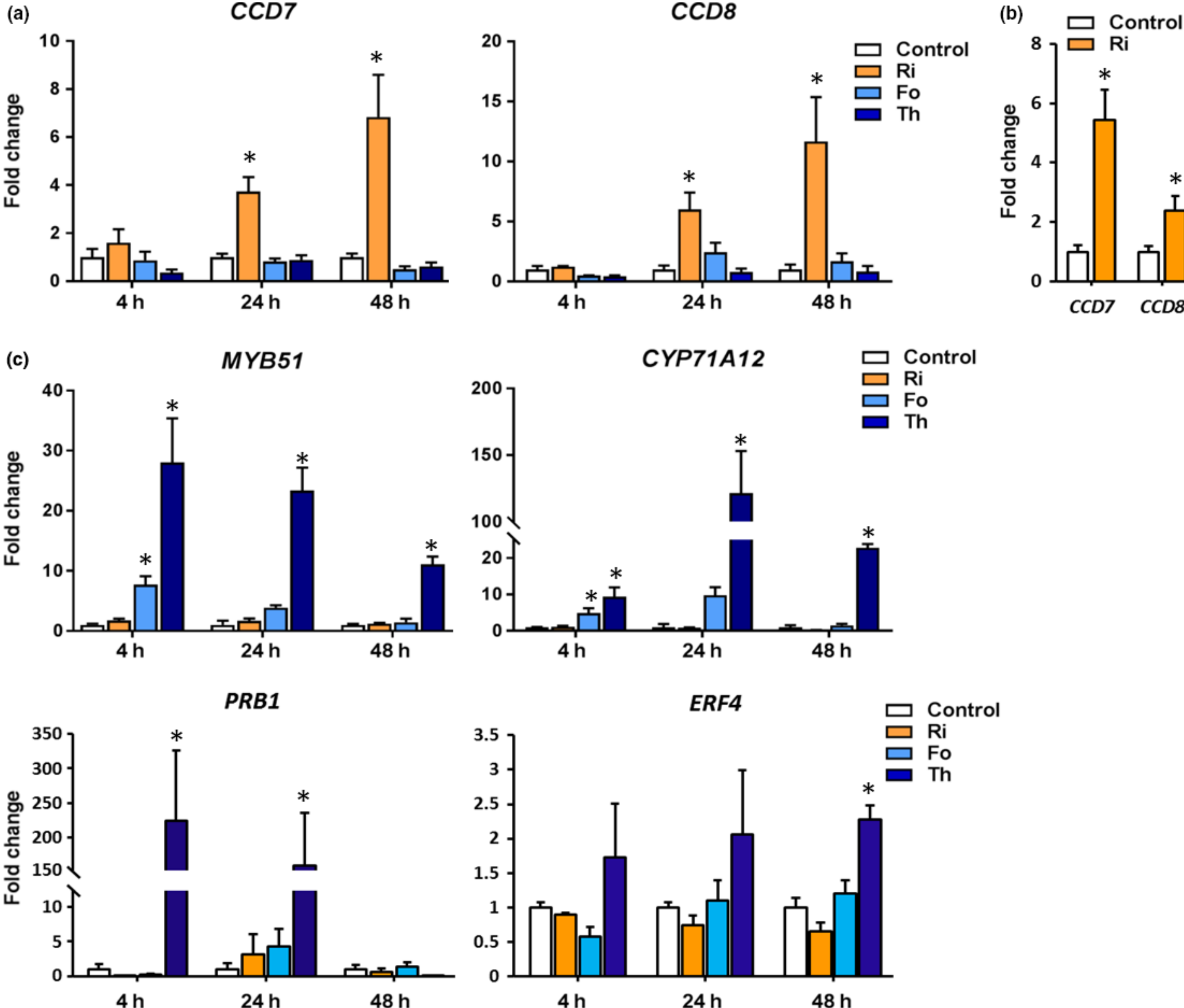

Fig. 1 Quantification of *CCD7*, *CCD8*, *MYB51*, *CYP71A12*, *PRB1* and *ERF4* transcript abundances in *Arabidopsis* roots during early interaction with *Rhizophagus*, *Fusarium* and *Trichoderma* fungi. (a) Relative expression of *Arabidopsis* strigolactone biosynthesis genes *CCD7* and *CCD8* after contact with *in vitro*-grown *Rhizophagus* (Ri), *Fusarium* (Fo) or *Trichoderma* (Th) mycelium. Gene expression was analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in 4-wk-old *Arabidopsis* roots at 4, 24 and 48 h after the initiation of contact with the fungal mycelium. (b) Relative expression of *Arabidopsis* strigolactone biosynthesis genes *CCD7* and CCD8 after contact with a suspension of Ri fungal structures (germinating spore exudates, spores and mycelium). Gene expression was analyzed by qRT-PCR in 4-wk-old *Arabidopsis* roots at 24 h after treatment with the suspension of *Rhizophagus* fungal structures. (c) Relative expression of *Arabidopsis* defense-related genes *MYB51*, *CYP71A12*, *PRB1* and *ERF4* after contact with *in vitro*-grown Ri, Fo or Th mycelium. Gene expression was analyzed by qRT-PCR in 4-wk-old *Arabidopsis* roots at 4, 24 and 48 h after the initiation of contact with the fungal mycelium. Values are means ± SE of six independent biological replicates. Each biological replicate consisted of pooled root systems from 20 (a, c) or five (b) 4-wk-old *Arabidopsis* seedlings. Relative expression was normalized to the *Arabidopsis* reference gene *At1g13320*. Asterisks indicate statistically significant differences compared with control plants per time point (Dunnett's test, $P \leq 0.05$).

are initially attracted to the roots of the nonhost *Arabidopsis*. However, to what extent strigolactones or other rhizodeposits of *Arabidopsis* function in this early interaction could not be established in this experimental setup.

Medicago-supported *Rhizophagus* colonizes *Arabidopsis* roots and reduces *Arabidopsis* shoot biomass

The results described earlier suggest an early signaling process between *Arabidopsis* and *Rhizophagus* which resembles the recognition processes described during the establishment of a functional AM symbiosis in host plants. However, in previous studies, Veiga *et al.* (2013) demonstrated that a *Rhizophagus* network supported by the host plants *Lolium multiflorum* or *Trifolium pratense* colonized *Arabidopsis* roots and inhibited the growth of this nonhost plant, pointing to antagonism. Using a similar microcosm setup (Fig. S2), we first verified whether a mycorrhizal network supported by the host plant *Medicago* has a similar antagonistic effect on growth. Optical and confocal

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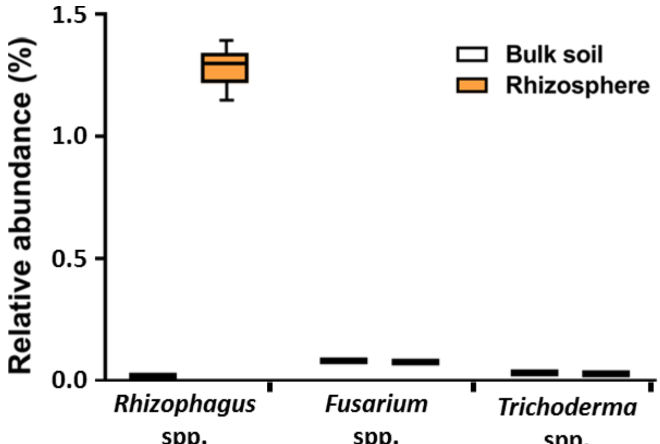

Fig. 2 Relative abundance of *Rhizophagus*, *Fusarium* and *Trichoderma* fungi in *Arabidopsis* rhizosphere. Relative abundance of *Rhizophagus* spp., *Fusarium* spp. and *Trichoderma* spp. fungi in the rhizosphere of *Arabidopsis* plants compared with unplanted bulk soil. Fungal abundances were estimated with PHYLOSEQ in shotgun metagenome sequencing data of microbial DNA in *Arabidopsis* root samples and bulk soil after classification of the reads with Kaiju. Values are means ± SE of three independent biological replicates. Only taxa with a relative abundance > 0.001% in at least one sample were included in the analysis.

imaging confirmed that *Rhizophagus* profusely colonized the roots of its host *Medicago*, resulting in the formation of arbuscules and vesicles that are characteristic of a functional symbiosis (Fig. 3a,e). The root surface of *Arabidopsis* roots grown in microcosms with a *Medicago*-supported AM fungal network was colonized by AM fungal hyphae. This frequently resulted in invasion of the *Arabidopsis* root cortex by *Rhizophagus* (Fig. 3b,c,f,g), confirming previous findings with the hosts *L. multiflorum* and *T. pratense* (Veiga *et al.*, 2013). However, the degree of root colonization in *Arabidopsis* was much lower (5 ± 1%, mean ± SE) than that observed in roots of *Medicago* (77 ± 3%). Despite this low colonization ratio, we observed the formation of typical mycorrhizal hyphopodia-like structures on the surface of *Arabidopsis* roots (Fig. 3b) and the presence of intraradical hyphae in the cortex of *Arabidopsis* roots (Fig. 3c,f,g). However, as opposed to *Medicago*, we typically did not observe the formation of arbuscules in the colonized roots of *Arabidopsis*. No fungal structures could be observed in *Arabidopsis* roots that were grown in *Rhizophagus*-inoculated soil but without the AM network supported by the host plant *Medicago* (Fig. 3d,h), confirming that a host-supported AM fungal network is required for colonization of *Arabidopsis* roots.

To corroborate the results obtained by light and confocal microscopy (Fig. 3), AM fungus colonization was further studied by analyzing the transcript abundance of the constitutively expressed *Rhizophagus* gene *GintrRNA* (Isayenkov *et al.*, 2004) in thoroughly washed roots. *GintrRNA* transcripts were detected in *Medicago* roots (Fig. 4a) and in *Arabidopsis* roots that were exposed to *Medicago*-supported *Rhizophagus* mycelium (Fig. 4b), further confirming that *Rhizophagus* colonized *Medicago* and *Arabidopsis* roots. Next, we analyzed the transcript abundances of *GintPT* (encoding the *Rhizophagus* high-affinity phosphate transporter; Maldonado-Mendoza *et al.*, 2001), *GintAMT2* (encoding the *Rhizophagus* high-affinity ammonium transporter; Pérez-Tienda *et al.*, 2011), *GintMST2* and *GintMST4* (encoding *Rhizophagus* high-affinity monosaccharide transporters; Helber *et al.*, 2011) in roots of *Medicago* and *Arabidopsis*. These genes are well-characterized markers for a functional AM symbiosis. The expression of the symbiosis-related genes was strongly upregulated in *Rhizophagus*-colonized *Medicago* roots (Fig. 4a), but not in AM fungus-colonized *Arabidopsis* roots (Fig. 4b), indicating that although *Medicago*-supported *Rhizophagus* is able to colonize *Arabidopsis* roots, the interaction does not result in a functional AM association.

To test the effect of AM fungi on growth of *Arabidopsis* in microcosms with and without *Medicago*-supported AM fungal networks, we measured the shoot FW. Fig. 5(a) shows that the *Medicago*-supported AM fungus significantly reduced *Arabidopsis* shoot biomass by *c.* 50%, compared with nonmycorrhizal *Arabidopsis* control plants. Although *Medicago* roots were heavily colonized by the AM fungus, no differences in shoot biomass between mycorrhizal and nonmycorrhizal *Medicago* plants were observed (Fig. 5a). We also measured the shoot FW of *Arabidopsis* plants that were grown in AM fungus-inoculated and noninoculated soil, without the presence of an AM fungal network supported by a host plant. Fig. 5(a) shows that, in absence of a host plant, *Rhizophagus* did not affect the shoot biomass of *Arabidopsis*. Collectively, these observations indicate that in the presence of an active AM fungal network provided by the host plant *Medicago*, *Rhizophagus* is able to colonize *Arabidopsis* roots. However, this interaction is not associated with the expression of symbiosis-related *Rhizophagus* genes *GintPT*, *GintAMT2*, *GintMST2* and *GintMST4*. Instead it comes with an ecological cost for the nonhost plant in terms of reduced shoot biomass production. To test whether strigolactones affect these responses in the later stages of the *Arabidopsis–Rhizophagus* interaction, we performed a bicompartment microcosm experiment using the *A. thaliana* strigolactone biosynthesis mutant *max1* as nonhost plant and *Medicago* as host plant. The results were similar to those observed for Col-0. Mutant *max1* roots became infected by *Rhizophagus* (Fig. S3a,c) and *Rhizophagus*-infected *max1* plants displayed a strong growth reduction (Fig. S3b). As in Col-0 roots, transcripts of the constitutively expressed *Rhizophagus GintrRNA* gene were detectable in *Rhizophagus*-infected *max1* roots, but the symbiosis-associated transcripts *GintPT* and *GintAMT2* were absent (Fig. S3c). Hence, strigolactones may play a role in early *Arabidopsis–Rhizophagus* interactions (Fig. 1), but after an extended growth period in a microcosm with a dense *Medicago* host-nursed mycorrhizal network, Col-0 and *max1* plants become similarly colonized by *Rhizophagus*.

Rhizophagus triggers plant defense responses in colonized *Arabidopsis* roots

To gain an insight into the molecular mechanisms underlying the differences between host and nonhost responses to *Rhizophagus* colonization, we compared the *Rhizophagus*-induced transcriptional changes in *Arabidopsis* and *Medicago* roots

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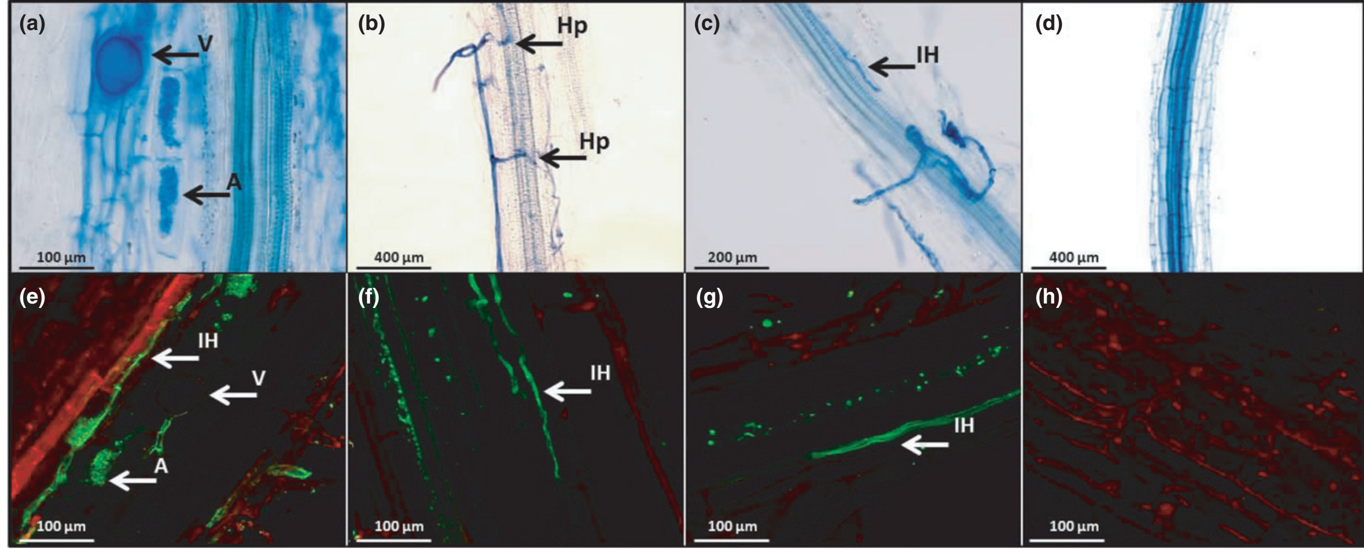

Fig. 3 Microscopic analysis of the colonization of *Medicago* and *Arabidopsis* roots by *Rhizophagus*. *Medicago*, *Rhizophagus* and *Arabidopsis* were grown together in the bicompartment microcosm system to allow the *Medicago*-nursed arbuscular mycorrhizal (AM) network to colonize *Arabidopsis* roots. Colonization of *Medicago* (a, e) and *Arabidopsis* (b, c, f, g) roots by *Rhizophagus* was visualized by light microscopy (a–d) and confocal microscopy (e–h). *Arabidopsis* plants were also grown in *Rhizophagus*-inoculated soil in the absence of *Medicago* (d, h). Arrows point to hyphopodia-like structures (Hp), intraradical hyphae (IH), arbuscules (A) and vesicles (V) formed in colonized *Medicago* and *Arabidopsis* roots. The experiment was repeated at least twice with similar results.

growing in the same AM fungal network. To this end, we performed an RNA-seq data analysis of the transcriptomes of *Arabidopsis* and *Medicago* roots in response to *Rhizophagus* colonization in the tripartite *Medicago–Rhizophagus–Arabidopsis* microcosm system. A principal component analysis of all root transcriptional changes showed that the mycorrhiza component explains the majority of the differences between the nonmycorrhizal and mycorrhizal *Medicago* samples (Fig. S4a). This was less clear for the *Arabidopsis* samples (Fig. S4b). Possibly, this is a result of the much lower frequency and less well developed *Rhizophagus* colonization on *Arabidopsis* roots, resulting in a larger variation in the transcriptional response to *Rhizophagus* than in the heavily colonized *Medicago* roots.

Analysis of the transcriptional profile of *Medicago* roots revealed 4953 DEGs, of which 3168 genes were upregulated and 1785 genes were downregulated in response to *Rhizophagus* inoculation (Table S2). Among the upregulated *Medicago* genes are *CCD7*, *CCD8*, *PT4* and *BCP1*, which have previously been found to be upregulated in response to AM fungi (Harrison *et al.*, 2002; Parádi *et al.*, 2010; Bonneau *et al.*, 2013). In agreement with the much lower root colonization frequency, the transcriptome changes in *Arabidopsis* roots in response to *Rhizophagus* inoculation were markedly smaller, but still consisted of 954 DEGs, 414 of which were upregulated and 540 of which were downregulated (Table S2). In line with our observations in the *in vitro* system (Fig. 1a), strigolactone biosynthesis genes *CCD7* and *CCD8* were upregulated (2.1- and 1.5-fold, respectively), albeit not significantly (P values 0.1 and 0.3 respectively).

To gain an insight into the differences in biological processes in *Medicago* and *Arabidopsis* roots that are likely to be influenced by AM fungal colonization, a GO enrichment analysis was performed on the set of DEGs that were identified in *Rhizophagus*-colonized *Medicago* and *Arabidopsis* roots (Table S3). Among the 20 most significantly overrepresented GO terms for the upregulated *Medicago* genes are several GO terms associated with carbohydrate metabolism and plant nutrient transport (Table 1a), reflecting the biological processes that are known to be involved in the establishment and maintenance of a functional AM symbiosis (Zouari *et al.*, 2014). By contrast, the 20 most significantly enriched GO terms for the upregulated *Arabidopsis* genes did not contain these GO terms. Instead, several defense-related GO terms were overrepresented, including GO terms related to sulfur compound (i.e. glucosinolate) biosynthesis and metabolism, salicylic acid biosynthesis and systemic acquired resistance (Table 1b). Among the upregulated defense-related genes are *FMO GS-OX3*, encoding the flavin-monooxygenase S-oxygenase 3 involved in glucosinolate biosynthesis (Kong *et al.*, 2016), and pathogenesis-related protein gene *PR-14*, encoding a lipid transfer protein (Sels *et al.*, 2008). These results suggest that in contrast to what we observed during early interaction stages in the *in vitro* system (Fig. 1b), in later interaction stages of the interaction, when *Rhizophagus* colonized the roots, the AM fungus activates plant

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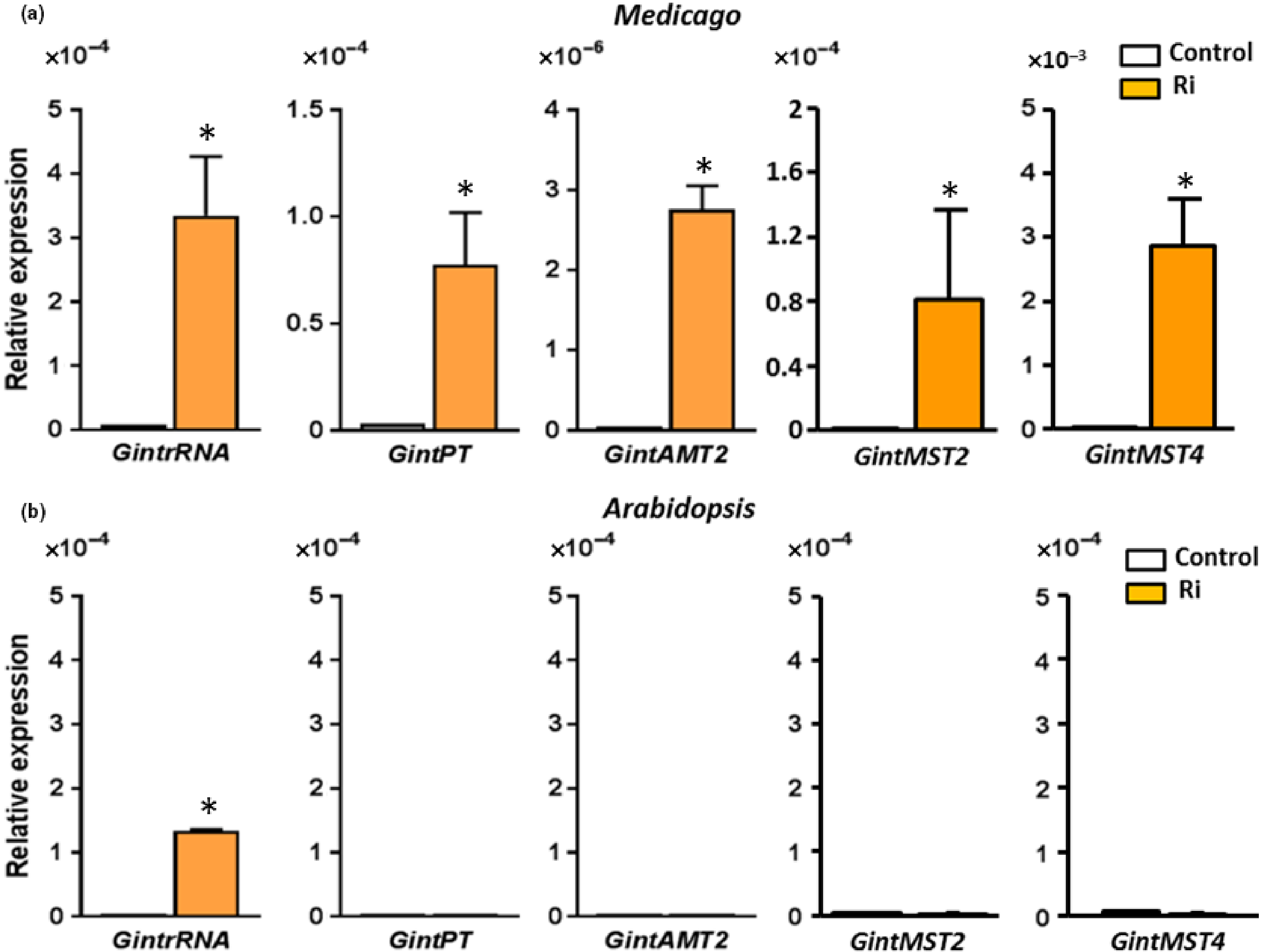

Fig. 4 Quantification of *Rhizophagus GintrRNA*, *GintPT*, *GintAMT2*, *GintMST2* and *GintMST4* transcript abundances in *Medicago* and *Arabidopsis* roots. *Medicago*, *Rhizophagus* and *Arabidopsis* were cocultivated in the bicompartment microcosm system to allow the *Medicago*-nursed arbuscular mycorrhizal (AM) network to colonize *Arabidopsis* roots. *Rhizophagus GintrRNA*, *GintPT*, *GintAMT2*, *GintMST2* and *GintMST4* transcript abundances were analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in colonized (Ri) *Medicago* (a) and *Arabidopsis* (b) roots. Whereas *GintrRNA* is a constitutively expressed gene *GintPT*, *GintAMT2*, *GintMST2* and *GintMST4* are well-characterized markers for a functional AM symbiosis. The relative expression of each *Rhizophagus* gene was normalized to constitutively expressed *Medicago* and *Arabidopsis* reference genes *MtEF1α* and *At1g13320*, respectively. Values are means ± SE of six independent biological replicates. Each biological replicate consisted of pooled root tissue from four *Medicago* (10 wk old) or 12 *Arabidopsis* (11 wk old) plants grown in the same bicompartment microcosm system. The asterisk indicates statistically significant differences between colonized (Ri) and noncolonized, mock-treated control plants (Student's *t*-test, $P \leq 0.05$). The experiment was repeated at least twice with similar results.

defenses, implying that it is ultimately recognized as an unwanted invader. Activation of root defenses can lead to growth–defense tradeoffs, resulting in inhibition of growth (Gomez-Gomez *et al.*, 1999; Stringlis *et al.*, 2018a), which could, at least in part, explain the growth reduction observed in *Arabidopsis* plants after colonization of the roots by *Rhizophagus*.

Root colonization by *Rhizophagus* induces systemic resistance in *Arabidopsis*

The observation that the GO term 'systemic acquired resistance' is overrepresented in the set of upregulated genes in *Rhizophagus*-colonized *Arabidopsis* roots prompted us to test whether host-supported AM fungi induce systemic pathogen resistance in *Arabidopsis*. To this end, we grew *Arabidopsis* plants in the bicompartment system alongside mycorrhizal or nonmycorrhizal *Medicago* plants, and inoculated them with the foliar necrotrophic fungus *B. cinerea*. Fig. 5(b) shows that *Arabidopsis* plants exposed to a *Medicago*-supported AM fungal network displayed a significant decrease in *B. cinerea* disease severity in comparison to noncolonized control plants. These results indicate that colonization of *Arabidopsis* roots by host-supported *Rhizophagus* can enhance plant immunity against pathogen infection.

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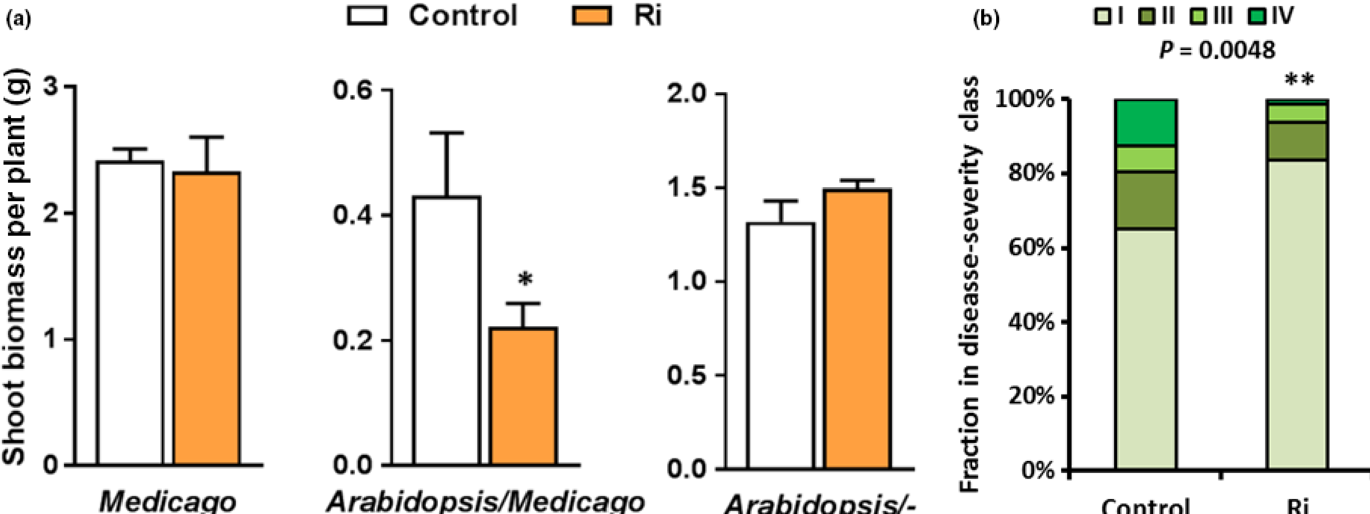

Fig. 5 Effect of *Rhizophagus*–root interaction on *Arabidopsis* growth and defense. (a) Shoot FW of *Medicago* and *Arabidopsis* plants cocultivated in the bicompartment microcosm system in *Rhizophagus*-inoculated soil (Ri) or mock-treated control soil. Values are means ± SE of six independent biological replicates. Each biological replicate consisted of pooled shoot tissue from four *Medicago* (10 wk old) or 12 *Arabidopsis* (11 wk old) plants grown in the same bicompartment microcosm. Asterisks indicate statistically significant differences (Student's *t*-test, $P \leq 0.05$). The experiment was repeated with similar results. (b) Quantification of disease symptoms of *Arabidopsis* plants grown in the presence or absence of the *Medicago*-nursed arbuscular mycorrhizal (AM) network, 72 h after inoculation of the leaves with *Botrytis cinerea*. Disease severity of inoculated leaves was scored in four classes, including restricted lesion (class I, 2–4 mm diameter), nonspreading lesion (class II, 5–6 mm diameter), spreading lesion (class III, 7–8 mm diameter), and severely spreading lesion (class IV, > 8 mm diameter). The percentage of leaves in each class was calculated per plant. Values are means of 10 independent biological replicates. Each biological replicate consisted of a single 9-wk-old *Arabidopsis* plant. Asterisks indicate statistically significant difference between Ri and noncolonized control plants (chi-squared test: **, $P < 0.01$).

Discussion

The molecular and physiological mechanisms behind the inability of nonhost plants to establish an AM symbiosis are poorly understood. The most probable explanation is that ancestors of nonhost plant species lost most of the symbiotic genes during evolution (Delaux *et al.*, 2014). However, contrary the current notion that nonhosts do not accommodate any AM fungi (Delaux *et al.*, 2014; Favre *et al.*, 2014; Bravo *et al.*, 2016), various members of the presumed nonhost family Brassicaceae (Brundrett, 2009), which lost the symbiotic genes (Delaux *et al.*, 2014), can under certain conditions still be endophytically colonized by AM fungi (Ocampo, 1986; De Mars & Boerner, 1995; Regvar *et al.*, 2003; Veiga *et al.*, 2013), and even occasionally form AM-like structures (Cosme *et al.*, 2018). Hence, it is important to investigate how nonhost plants interact with AM fungi. We observed that when *Arabidopsis* was grown in a soil collected from its natural habitat, the roots of this nonhost plant increased the abundance of *Rhizophagus* spp. in its rhizosphere microbiome, compared with the microbiome of the control bulk soil without *Arabidopsis* roots (Fig. 2), suggesting that there are previously unconsidered interactions between AM fungi and nonhost plants.

Arabidopsis seems to specifically detect the AM fungus *Rhizophagus* in initial stages of the interaction

During the presymbiotic stage of AM symbiosis, both partners communicate through the exchange of diffusible molecules (Gutjahr & Parniske, 2013). Spores of AM fungi are only capable of limited growth in the absence of a host plant. Plant roots excrete specific metabolites that advertise their presence in the soil and stimulate presymbiotic fungal growth before colonization of the root (Nadal & Paszkowski, 2013). Plant strigolactones have been identified as major contributors during plant–AM fungi communication in the presymbiotic stages (Akiyama *et al.*, 2005). Interestingly, the strigolactone biosynthesis pathway is also present in the nonhost plant *Arabidopsis* (Delaux *et al.*, 2014; Fig. S5). We found that the expression of the *Arabidopsis* strigolactone biosynthesis genes *CCD7* and *CCD8* was induced in the nonhost roots after exposure of the roots to the AM fungus (Fig. 1a,b). These results suggest that the nonhost plant *Arabidopsis* detects the presence of the AM fungus and responds like AM hosts by increasing the expression of the strigolactone biosynthesis genes *CCD7* and *CCD8*. This observation is consistent with earlier findings that, during spore germination, hyphal branching and fungal attachment to the roots, there is no obvious indication that AM fungi can discriminate between host and nonhost plants (Tester *et al.*, 1987; Giovannetti & Sbrana, 1998). Interestingly, induction of *CCD7* and *CCD8* by *Rhizophagus* is not a general response of *Arabidopsis* to fungal detection, because the pathogenic fungus *Fusarium* and the beneficial endophyte *Trichoderma* did not induce the expression of these genes (Fig. 1a). Together, these results suggest that the early induction of strigolactone biosynthesis genes in response to AM fungi is still conserved in *Arabidopsis*, probably reflecting that this nonmycorrhizal plant evolved from mycorrhizal ancestors.

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Table 1 Gene ontology (GO) enrichment terms associated with upregulated differentially expressed genes on *Medicago* and *Arabidopsis* roots after *Rhizophagus* colonization.

GO terms, ID (*Medicago*)	GO terms, category	$Log_{10}P$
(a)		
GO:0006412	Translation	−57.7375
GO:0010035	Response to inorganic substance	−23.4401
GO:0044710	Single-organism metabolic process	−16.1656
GO:0044711	Single-organism biosynthetic process	−15.1385
GO:0044281	Small molecule metabolic process	−14.9586
GO:0005975	Carbohydrate metabolic process*	−14.3307
GO:0016114	Terpenoid biosynthetic process*	−13.0501
GO:0055114	Oxidation-reduction process	−12.5302
GO:0009628	Response to abiotic stimulus	−12.129
GO:0044712	Single-organism catabolic process	−11.5834
GO:0006970	Response to osmotic stress	−11.4023
GO:0019318	Hexose metabolic process*	−11.3382
GO:0009607	Response to biotic stimulus	−9.5243
GO:0006820	Anion transport	−7.5719
GO:0046394	Carboxylic acid biosynthetic process*	−7.0482
GO:0098542	Defense response to other organism	−7.0315
GO:0009853	Photorespiration	−6.0511
GO:0071705	Nitrogen compound transport*	−5.5969
GO:1901564	Organonitrogen compound metabolic process*	−5.3019
GO:1901334	Lactone metabolic process	−5.0405

GO terms, ID (*Arabidopsis*)	GO terms, category	$Log_{10}P$
(b)		
GO:0019684	Photosynthesis, light reaction	−30.2596
GO:0006790	Sulfur compound metabolic process*	−20.9626
GO:0044711	Single-organism biosynthetic process	−19.7645
GO:0035304	Regulation of protein dephosphorylation	−19.1273
GO:0044272	Sulfur compound biosynthetic process*	−18.7328
GO:0006364	rRNA processing	−18.6819
GO:0006098	Pentose-phosphate shunt	−17.9914
GO:0009070	Serine family amino acid biosynthetic process	−16.5243
GO:0009657	Plastid organization	−16.0535
GO:0009628	Response to abiotic stimulus	−14.6576
GO:0009862	Systemic acquired resistance, salicylic acid-mediated signaling pathway*	−13.585
GO:0010310	Regulation of hydrogen peroxide metabolic process	−12.5143
GO:0010114	Response to red light	−12.02
GO:0000165	MAPK cascade	−10.8761
GO:0043085	Positive regulation of catalytic activity	−10.5302
GO:0043900	Regulation of multiorganism process	−9.983
GO:0009697	Salicylic acid biosynthetic process*	−9.7959
GO:0046148	Pigment biosynthetic process	−9.7747
GO:0019252	Starch biosynthetic process	−9.1141
GO:0015995	Chlorophyll biosynthetic process	−6.3645

The 20 most significant overrepresented GO terms (biological process), associated with upregulated DEGs, on *Medicago* (a) and *Arabidopsis* (b) roots after *Rhizophagus* colonization were analyzed by PLAZA and REVIGO software. Black asterisks indicate GO terms associated with carbohydrate metabolism and plant nutrient transport. Red asterisks indicate GO terms related to sulfur compound biosynthesis, salicylic acid biosynthesis and systemic acquired resistance. Significance was plotted as a red–green color scale with green indicating low significance and red high significance.

Rhizophagus colonizes the nonhost *Arabidopsis* endophytically, without establishing a functional symbiosis

By using a similar setup as Veiga *et al.* (2013) (Fig. S2), we explored whether *Arabidopsis* was able to establish a functional symbiosis when an active mycelial network was nursed by the AM host *Medicago*. *Rhizophagus* was indeed able to colonize the root cortex of *Arabidopsis*, but only when the AM network was supported by *Medicago* (Fig. 3). We found typical intraradical aseptated hyphae inside the root cortex and few hyphopodia-like structures on the surface of *Arabidopsis* roots. These findings were further supported by the observation that transcripts of the constitutively expressed *Rhizophagus* gene *GintrRNA* (Fig. 4b) accumulated in *Arabidopsis* roots grown in soil with an active *Medicago*-supported mycorrhizal network.

Although *Arabidopsis* roots were colonized by *Rhizophagus*, we did not detect arbuscules, confirming previous findings (Veiga *et al.*, 2013). The absence of arbuscules in the cortex of *Arabidopsis* indicates that the interaction between AM fungi and *Arabidopsis* does not represent a typical AM symbiosis. To verify this, we checked the expression of the AM symbiosis marker genes *GintPT*, *GintAMT2*, *GintMST2* and *GintMST4*. Although strongly activated during colonization of *Medicago* roots (Fig. 4a), these marker genes remained mute in *Rhizophagus* when interacting with *Arabidopsis* roots (Fig. 4b), further corroborating the absence of AM functionality. Collectively these results strengthen previous finding by Veiga *et al.* (2013) showing the ability of *Rhizophagus* to colonize *Arabidopsis* roots endophytically, and further demonstrating the absence of a fully functional AM symbiosis in this interaction.

Host-supported *Rhizophagus* suppresses *Arabidopsis* growth but stimulates immunity

Growth promotion is one of the multiple benefits that the AM fungi usually provide to their host partners. However, in our study we observed a strong growth reduction in shoot biomass of *Arabidopsis* plants that were colonized by the AM fungus *Rhizophagus*, even though the amount of root colonization was only 5% (Fig. 5a). Interestingly, this growth reduction was observed only when the AM fungal network was nursed by the AM host plant, and thus leading to fungal colonization of the *Arabidopsis* roots. It is known that AM fungi are able to interact simultaneously with several partners and exchange their resources in highly complex partnerships, with positive, negative or neutral outcomes of the individual partners (Newman & Reddell, 1988; Van der Heijden & Horton, 2009; Werner *et al.*, 2014). Therefore, the growth reduction found in *Arabidopsis* might, in addition to defense activation, be related to such a negative outcome. The mycorrhizal network may acquire nutrients from the soil near *Arabidopsis* roots and store them in the mycelial network or deliver them to *Medicago*, the host plant maintaining the mycorrhizal network. Consequently, reduced nutrient availability might explain the growth reduction of *Arabidopsis*. However, no significant differences were found in the phosphorus, N or C content of *Arabidopsis* plants colonized by *Rhizophagus* compared with

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noncolonized control plants (Table S4). These results do not indicate that a *Medicago*-nursed *Rhizophagus*-mediated reduction of nutrient availability is responsible for the growth reduction observed in *Rhizophagus*-colonized *Arabidopsis*.

A second explanation for the growth reduction observed in *Rhizophagus*-colonized *Arabidopsis* may be related to the possibility that upon colonization of the *Arabidopsis* roots, *Rhizophagus* is detected as an unwanted invader. After transfer of *Arabidopsis* roots to *Rhizophagus* mycelium, we did not observe activation of the early root immunity genes *MYB51*, *CYP71A12*, *PRB1* and *ERF4*, even though these genes were strongly activated in response to the root pathogen *Fusarium* or the beneficial root fungus *Trichoderma* (Fig. 1c), perhaps reflecting that in early interaction stages, AM fungi are not detected as antagonists. However, RNA-seq data analysis of later interaction stages of the *Rhizophagus*-colonized *Arabidopsis* roots revealed signatures of defense activation (Table 1b), corroborating the possibility that host-supported *Rhizophagus* induces costly defenses that reduce plant growth. Such induced defenses may limit *Rhizophagus* invasion, but also confer systemic resistance against pathogen infection, as exemplified by our observation that *Rhizophagus*-colonized *Arabidopsis* plants display enhanced resistance against the foliar pathogen *B. cinerea* (Fig. 5b). Modulation of local and systemic plant defense responses have frequently been described to occur in mycorrhizal plants (Jung *et al.*, 2012). As a result, mycorrhizal plants can become primed for enhanced defense, resulting in a more efficient activation of defense mechanisms in response to attack by potential enemies (Martínez-Medina *et al.*, 2016). However, whether the observed enhanced resistance in *Rhizophagus*-colonized *Arabidopsis* plants is based on the same phenomenon remains to be elucidated.

In sum, our results provide evidence that the presymbiotic interaction of nonhost *Arabidopsis* plants with host-supported mycorrhizal fungi resembles at least some of the processes observed during the presymbiotic dialogue in host plant–AM fungus interactions. In the later stages of the interaction, the AM fungus colonizes the root cortex without forming a functional AM symbiosis. Instead, defense responses are activated that are associated with a reduction of plant growth and enhanced resistance against pathogen infection. This study highlights the multifaceted functions of mycorrhizal fungi in nature and sheds new light on the role that mycorrhizal fungi can play in plant communities with both AM host and nonhost plants.

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Author contributions

IF, SCMvW, MJP, CMJP and MGAvdH planned and designed the research. IF, IAS, KY and RvJ performed experiments and analyzed the data. IF, MC, IAS, CMJP and MGAvdH wrote the manuscript. CMJP and MGAvdH contributed equally to this work.

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Supporting Information

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Fig. S1 *In vitro* system designed to test the effects of *Rhizophagus*, *Fusarium* and *Trichoderma*, on *Arabidopsis* root responses.

Fig. S2 Schematic representation of the *Medicago–Rhizophagus–Arabidopsis* bicompartment microcosm system.

Fig. S3 Effect of root colonization by *Rhizophagus* on the *Arabidopsis* strigolactone biosynthesis mutant *max1*.

Fig. S4 Principal component analysis and clustering of RNA-seq profiles of *Medicago* and *Arabidopsis* roots colonized by the AM fungus *Rhizophagus*.

Fig. S5 Occurrence of symbiosis genes in host and nonhost plants.

Table S1 Primers used for gene expression analysis in this study.

Table S2 Differentially expressed genes (DEGs) in *Medicago* and *Arabidopsis* roots colonized by the AM fungus *Rhizophagus* in comparison with noncolonized control plants.

Table S3 Gene ontology (GO) terms (based on biological process) that are significantly enriched in the up- and the downregulated sets of DEGs in *Medicago* and *Arabidopsis* roots colonized by the AM fungus *Rhizophagus* in comparison to noncolonized control plants.

Table S4 Nutrient content in *Medicago* and *Arabidopsis* plants colonized by *Rhizophagus* (Ri) compared with noncolonized control plants.

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