Short title: *Medicago*'s responses to variable benefit AM fungi

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Physiological and transcriptomic response of *Medicago truncatula* to high and low benefit mycorrhizal fungi

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One sentence summary: Colonization with high- and low-benefit arbuscular mycorrhizal fungi leads to distinct physiological and transcriptomic changes in the roots and shoots of *Medicago truncatula*.

Footnotes:

List of Author contributions. H.B. and S.S. conceived the original research plans; H.B. and A.K. designed the experiments; A.K. performed the long-term experiment and J.Y. the short-term experiment, K.R.C. and K.G. conducted the transcriptome analysis, and P.P. and G.S. completed the stable isotope analysis; K.R.C., A.K., K.G., J.Y. and P.P. analyzed the data; K.R.C. wrote the article with input from all the authors; H.B. and S.S. supervised and completed the writing. H.B. agrees to serve as the author responsible for contact and ensures communication.

Responsibilities of the Author for Contact. The corresponding author, Heike Bücking, ensures that all scientists who have contributed substantially to the conception, design, or execution of the work are included as authors, and that all authors have agreed to the list of authors and to the identified contributions of the authors.

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi provide their host plants with greater access to limited mineral nutrients, but the amount they provide can be variable. Here, we evaluated the capacity of the high-benefit fungus *Rhizophagus irregularis* 09 and the low-benefit fungus *Glomus aggregatum* 165 to transfer nitrogen and phosphorus to the host plant *Medicago truncatula*, and identified putative molecular mechanisms regulating the physiological response of the host to these fungi. *R. irregularis* led to an increase in plant biomass and transferred more nitrogen and phosphate to the host than *G. aggregatum*. This increase was linked to elevated expression of known mycorrhiza-induced phosphate (*PT8*), ammonium (*AMT2;3*), and nitrate (*NPF4.12*) transporters in the roots, as well as the putative ammonium transporter *NIP1;5*. *R. irregularis* also stimulated the expression of photosynthesis related genes in the shoot and the upregulation of the mycorrhiza-induced sugar transporter *SWEET1.2* and the lipid biosynthesis gene *RAM2* in the roots, which is indicative of increased carbon flux to this fungus. In contrast, *G. aggregatum* induced biotic stress defense response genes (e.g., *Medtr4g120760* and *Medtr8g096900*) in the shoots, and several genes associated with the GO term “response to water deprivation” in the roots of *M. truncatula*. This could indicate that the host perceives colonization by the low-benefit fungus as pathogen attack, or that *G. aggregatum* is more effective than *R. irregularis* at priming host defense responses. Our findings reveal novel insights into the molecular mechanisms by which host plants reward high-but sanction low-benefit arbuscular mycorrhizal symbionts.

INTRODUCTION

Nitrogen (N) and phosphorus (P) are the main nutrients that limit plant growth in natural and agricultural ecosystems (LeBauer and Treseder, 2008; Vitousek et al., 2010). Conventional management practices typically address both N and P limitations through the application of chemical fertilizers, but fertilizer production, transportation, and application are both economically and environmentally expensive (Havlin et al., 2014). Implementing more sustainable methods to mitigate nutrient deficiencies in crops is essential to prevent continued environmental degradation but still meet the nutritional demands of a growing human population.

To overcome nutrient limitations, plants have evolved the capacity to form alliances with symbiotic, root-associated microbes (Martin et al., 2017). The most widespread symbiosis is the arbuscular mycorrhizal (AM) symbiosis formed by 72% of all known plant species and a relatively small number of fungal species in the Glomeromycotina (Spatafora et al., 2016; Brundrett and Tedersoo, 2018). AM fungi provide the host plant with mineral nutrients, such as P and N, and improve the resistance of plants against abiotic and biotic stresses (Bücking and Kafle, 2015; Kafle et al., 2019). Under P deficiency, plants increase the production of strigolactones, a class of phytohormones that also serve as signaling molecules perceived by AM fungi (Yoneyama et al., 2007). Strigolactones are exuded into the rhizosphere through the ATP-binding cassette transporter PDR1 (Kretzschmar et al., 2012). AM fungal perception of strigolactones triggers spore germination, hyphal branching, and the production of short-chain chitin oligomers (Besserer et al., 2008; Genre et al., 2013). These, along with lipo-chitoooligosaccharides (Maillet et al., 2011; Rush et al., 2020), comprise fungal “Myc factors” that prime the host plant for colonization by activating the “common symbiosis signaling pathway” (CSSP; reviewed in MacLean et al., 2017).

Activation of the CSSP by AM fungi culminates in the expression of several transcription factors required for the development and regulation of the AM symbiosis (Pimprikar and Gutjahr, 2018). Following root penetration, AM hyphae proliferate both intra- and intercellularly and form highly branched nutrient exchange structures called arbuscules in cortical cells (Park et al., 2015). Surrounding the arbuscule is a plant-derived periarbuscular membrane (PAM) that is enriched in AM-induced transporters that facilitate reciprocal nutrient exchange between the fungus and the plant (Garcia et al., 2016). In *Medicago truncatula*, three AMT2 family ammonium transporters (AMT2;3, AMT2;4, and AMT2;5; Breuillin-Sessoms et al., 2015) and the phosphate transporters *PT4* (Javot et al., 2007) and *PT8* (Breuillin-Sessoms et al., 2015) are involved in the uptake of N and P from the periarbuscular space between the fungal plasma membrane and the PAM. In exchange, host plants transfer about 20% of their photosynthetically derived carbon to their fungal partners, and these high carbon costs force host plants to strongly regulate the carbon flux to its fungal symbionts (Wright et al., 1998). The host plant releases lipids and sugars into the mycorrhizal interface likely via the putative lipid transporters *STR* and *STR2* (Zhang et al., 2010; Roth and Paszkowski, 2017) and the bidirectional sugar transporter *SWEET1.2* (formerly *SWEET1b*, An et al., 2019; Doidy et al., 2019).

AM fungi differ in the benefits that they provide for their host plant. The cooperative AM fungus *Rhizophagus irregularis* 09 (Ri09) transfers more N and P to its host plant and contributes to higher mycorrhizal growth responses than the less-cooperative fungus *Glomus aggregatum* 165 (Ga165; Fellbaum et al., 2014). Symbiosis with Ga165 is also more costly for the host plant (measured as carbon costs per P transferred) than with Ri09, and *M. truncatula* preferentially allocates more carbon to Ri09 than to Ga165 (Kiers et al., 2011). Collectively, these studies suggest that carbon to nutrient exchange processes are driven by biological market dynamics, but the

molecular mechanisms that regulate these processes are largely unknown. Here, we examine the physiological and transcriptomic responses of the host plant *M. truncatula* to colonization by Ri09 or Ga165 under N and P limitation to gain further insight into the molecular processes that trigger the differential response of a host plant to high- and low benefit AM fungal partners.

RESULTS

Colonization by *Rhizophagus irregularis* 09 leads to higher plant biomass and phosphorus and nitrogen uptake than *Glomus aggregatum* 165

We used a dual-compartment pot system to compare the overall benefit that Ga165 and Ri09 provided to *M. truncatula* (**Supplemental Fig. S1**). The colonization rates for both fungi were comparable and exceeded 80% (**Supplemental Fig. S2**). Compared to non-mycorrhizal (NM) plants, Ri09 colonization led to a higher shoot and root biomass, whereas Ga165 provided no growth benefits (**Fig. 1A-B**). Both fungi increased P content (**Fig. 1C-D**) and concentration (**Supplemental Fig. S3**) in the shoots and roots of their host, but Ri09 transferred more P than Ga165. Nitrogen-15 (^{15}N) enrichment levels in the roots and shoots of NM plants did not exceed natural abundance levels (**Fig. 1E-F**), which confirms that there was no mass flow of ^{15}N from the HC into the RC. Both fungi transferred ^{15}N from the HC to their host, but Ga165 only increased root ^{15}N enrichment, whereas Ri09 increased both root and shoot ^{15}N enrichment. Overall, these data indicate that compared to Ga165, Ri09 promotes increased growth by providing its host plant with greater access to both P and N.

Colonization by either fungus alters global gene expression more dramatically in the roots than in the shoots of *Medicago truncatula*

Figure 1

Figure 1. Biomass and nutrient content of *Medicago truncatula* plants colonized by different arbuscular mycorrhizal fungi. Shown are dry weight (A,B), phosphorus content (C,D), and % nitrogen-15 enrichment (E,F) of roots (left) and shoots (right). The plants were inoculated with either *Glomus aggregatum* 165 (Ga165) or *Rhizophagus irregularis* 09 (Ri09) and compared to non-mycorrhizal (NM) control plants. Bars represent the mean of each treatment (n = 5) ± SEM. Different letters on the bars indicate statistically significant ($p \leq 0.05$) differences based on one-way ANOVA and LSD test. F statistics and p-values are shown in Supplemental Table S1.

To identify potential molecular mechanisms responsible for the observed physiological responses, we used RNA sequencing (RNA-seq) to evaluate global gene expression patterns in roots and shoots of mycorrhizal and non-mycorrhizal plants. Analysis of the root data revealed distinct, treatment-specific hierarchical clustering among biological replicates (n=3, Ga165 and NM; n=4, Ri09) using a dendrogram, multidimensional scaling, and principal component analyses (**Supplemental Fig. S4**). In contrast, analysis of the shoot data showed no distinct clustering among biological replicates (n=4, Ga165 and NM; n=3, Ri09) within each treatment (**Supplemental Fig. S5**), suggesting that only in the roots, global gene expression is driven by the fungal partner. Consequently, the overall number of significant differentially expressed genes (DEGs) was lower in the shoots than in the roots (**Supplemental Fig. S6**).

Both shared and distinct sets of genes are differentially regulated in *Medicago truncatula* by colonization with either fungus

To identify key differences in the response of the host plant to both fungal species, we sorted all DEGs from each treatment and used an arbitrary threshold of $\log_2(\text{fold-change}) > 2$ and a q-value < 0.05 (**Fig. 2A**). In the roots, this threshold revealed that 364 genes were upregulated in roots colonized by either fungus, and 197 or 165 genes were upregulated by colonization with only Ga165 or Ri09, respectively (**Fig. 2B**). Ga165 and Ri09 colonization led to the downregulation of 41 shared genes, and 172 and 77 unique genes, respectively. The clustering of DEGs allowed us to identify specific groups of similarly expressed genes (**Fig. 2C**). Clusters A and B are comprised of genes that were strongly downregulated in mycorrhizal roots, cluster C1 contains genes that were upregulated in mycorrhizal roots, clusters C2 and D include genes primarily upregulated by Ga165 colonization, and cluster C3 contains genes exclusively upregulated by Ri09 colonization.

Figure 2

Figure 2. Significantly differentially expressed genes in roots (A–C) and shoots (D–F) of *Medicago truncatula* colonized by different AM fungi. (A, D) Number of significantly differentially expressed genes (DEGs; $\log_2[\text{fold-change}] > 2$ and a $q\text{-value} < 0.05$) in plants colonized by *Glomus aggregatum* 165 (Ga165) or *Rhizophagus irregularis* 09 (Ri09) compared either to non-mycorrhizal (NM) control plants or to each other. (B, E) Venn diagrams of significantly upregulated (yellow) and downregulated (blue) DEGs unique to Ga165 and Ri09-colonized plants or shared by both. (C, F) Heat maps of fragments per kilobase of transcript per million mapped reads (FPKM) values + 1 for all significant DEGs from each treatment. Gene clusters are assigned based on second-order branching. Upregulated genes are shown in yellow and downregulated genes in blue with intensity based on row z-score. All DEGs in each plot for roots and shoots are listed in Supplemental Files 1 and 2, respectively.

Although fewer genes were differentially expressed in the shoots, many were above our applied threshold (**Fig. 2D**). We found that 48 and 14 genes were up, and 13 and 27 genes were downregulated in the shoots of Ga165 and Ri09-colonized plants, respectively (**Fig. 2E**). In contrast to the roots, clustering of DEGs based on expression patterns in the shoots (**Fig. 2F**) revealed that only a small number of genes were upregulated (cluster C1) or downregulated (cluster A) in mycorrhizal plants. In addition, some genes were only downregulated by Ga165 (cluster B) or by Ri09 colonization (cluster D), while others were primarily upregulated by Ga165 colonization (cluster C2). Collectively, these results demonstrate that compared to shoots, gene expression patterns in roots respond more strongly to colonization with different AM fungi, and that both fungi lead to distinct differences in both root and shoot transcriptome profiles.

Genes important for AM symbiosis are more strongly upregulated by *Rhizophagus irregularis* 09 than *Glomus aggregatum* 165

We conducted a targeted analysis of the root RNA-seq data by first evaluating the expression of key AM symbiosis genes (**Supplemental Table S2**). Among six core strigolactone biosynthesis or exporter genes (**Fig. 3A**), we observed that two (*DXS2* and *D27*) were upregulated in AM roots compared to the NM control; one (*MAX1a*) was only upregulated in Ri09-colonized roots, while three (*CCD7*, *CCD8-1* and *PDR1a*) were not affected. Two GRAS transcription factors (*NSP1* and *NSP2*) that regulate strigolactone biosynthesis and other symbiosis genes were also upregulated, particularly in Ri09-colonized roots. The combined increased expression of *NSP1*, *NSP2*, *CCD7*, and *MAX1a* (**Fig. 3B-E**) suggests that Ri09 more strongly induces strigolactone biosynthesis in the roots than Ga165.

Figure 3

Figure 3. Expression patterns of genes in the strigolactone biosynthesis pathway. (A) Diagram of the strigolactone biosynthesis pathway and the relative expression levels of genes in *Medicago truncatula* that encode components of the pathway shown as fragments per kilobase of transcript per million mapped reads (FPKM; blue=low, yellow=high) in roots from non-mycorrhizal (NM) plants or plants colonized by either *Glomus aggregatum* 165 (Ga165) or *Rhizophagus irregularis* 09 (Ri09). The expression patterns (FPKM) for four specific genes upregulated by Ri09-colonization are also shown, including (B) *More Axillary Growth 1a* (MAX1a), (C) *Carotenoid Cleavage Dioxygenase 7* (CCD7), (D) *Nodulation Signaling Pathway 1* (NSP1) and (E) *NSP2* in NM roots (n=3) and roots inoculated with Ga165 (n=3) or Ri09 (n=4). Bars represent the mean of each treatment $\pm$ SEM. Significant differences (q-value < 0.05) in FPKM values between treatments were determined as part of the CuffDiff2 analysis and are indicated using different letters (see Supplemental Table S2). Abbreviations in A: GAP, glyceraldehyde 3-phosphate; DXS2, 1-deoxyxylulose 5-phosphate synthase2; MEP, 2-methyl-erythritol-4-phosphate; GGDP, geranylgeranyl pyrophosphate; D27, Dwarf27; and PDR1a, pleiotropic drug resistance 1a.

Among the CSSP genes, the lipochitooligosaccharide receptor *NFP* was downregulated and *PUBL1*, a negative regulator of AM symbiosis, was upregulated in roots colonized by either fungus (**Supplemental Table S2**). None of the other 13 analyzed CSSP genes were affected. Downstream of the CSSP, we evaluated the expression of 21 genes (**Supplemental Table S3**), including: seven AM-induced transcription factors that regulate arbuscule development, function, or degradation — *DIP1*, *RAD1*, *RAM1*, *MIG1/2/3*, and *MYB1*; three cellular remodeling genes — *EXO70L*, *SYPI32A*, and *VAPYRIN*; two phosphate transporters (*PT4* and *PT8*); three ammonium transporters (*AMT2;3*, *AMT2;4*, and *AMT2;5*); the putative lipid transporters *STR* and *STR2* and three lipid biosynthesis genes — *RAM2*, *FatM*, and *WRI5a*; and the sugar transporter *SWEET1.2*. Almost all these genes were upregulated by both AM fungal species; however, six were substantially more upregulated by Ri09, including: *RAD1*, *MYB1*, *PT8*, *AMT2;3*, *RAM2*, and *SWEET1.2* (**Fig. 4A-F**). Together with the physiological results, the increased upregulation of transporter genes in Ri09-colonized roots is suggestive of greater nutrient exchange activity across the PAM in these roots.

Gene ontology enrichment differs in the roots of *Medicago truncatula* when colonized by *Glomus aggregatum* 165 or *Rhizophagus irregularis* 09

We employed gene ontology (GO) enrichment analysis to identify cellular components, molecular functions, and/or biological processes that were significantly enriched among genes that are only upregulated (**Supplemental Fig. S7**) or downregulated (**Supplemental Fig. S8**) in AM roots. Although we found many enriched GO terms, we only focus here on those associated with biotic or abiotic stress and nutrient transport due to the key role that AM fungi play for these processes. Among upregulated genes, the GO term “response to water deprivation” (GO:0009414; 6 genes)

Figure 4

Figure 4. Relative expression of six genes in the roots of *Medicago truncatula* after colonization with different AM fungi. Expression of (A) *Required for Arbuscule Development 1* (*RAD1*), (B) *MYB-like Transcription Factor 1* (*MYB1*), (C) *Phosphate Transporter 8* (*PT8*), (D) *Ammonium Transporter 2;3* (*AMT2;3*), (E) *Reduced Arbuscular Mycorrhization 2* (*RAM2*), and (F) *Sugars Will Eventually Be Exported Transporter 1.2* (*SWEET1.2*) are shown for non-mycorrhizal plants (NM, n=3) and plants inoculated with *Glomus aggregatum* 165 (Ga165, n=3) or *Rhizophagus irregularis* 09 (Ri09, n=4). Expression levels are shown as fragments per kilobase of transcript per million mapped reads (FPKM). Bars represent the mean of each treatment $\pm$ SEM. Significant differences (q-value < 0.05) in FPKM values between treatments were determined as part of the CuffDiff2 analysis and are indicated using different letters (see Supplemental Table S3).

was enriched in Gal65-colonized roots (**Supplemental Fig. S7A**), while “defense response” (GO:0006952; 11 genes) was enriched in Ri09-colonized roots (**Supplemental Fig. S7B**). The GO terms “integral membrane components” (GO:0016021; 104 genes) and “modification of morphology or physiology of other organism” (GO:00335821; 3 genes) were enriched by both fungi (**Supplemental Fig. S7C**). Among down-regulated genes, the GO term “root hair elongation” (GO:0008970, 3 genes) was enriched in Gal65-colonized roots (**Supplemental Fig. S8A**).

Colonization with *Glomus aggregatum* 165 and *Rhizophagus irregularis* 09 differentially affect the expression of transporters

Among upregulated genes, the GO terms “regulation of anion transmembrane transport” (GO:1903959; 2 genes) and “anion transmembrane transporter activity” (GO:0008509; 5 genes) were enriched in Ri09-colonized and AM-colonized roots, respectively (**Supplemental Fig. S7B-C**). For downregulated genes, five GO terms related to transport were enriched in Gal65-colonized roots (**Supplemental Fig. S8A**), but only two transport-related GO terms were enriched in Ri09-colonized roots (**Supplemental Fig S8B**). The GO term “transmembrane transport” (GO:0055085; five genes) was enriched by AM colonization (**Supplemental Fig. S8C**).

Given the dramatic difference in GO terms related to transport among Gal65 and Ri09-colonized roots, we filtered all gene annotations containing the term “transport” from the *M. truncatula* JCVI 4.0v2 annotation file (Tang et al., 2014) and found 259 genes that were significantly differentially regulated in mycorrhizal roots (**Supplemental File 4**). Based on their function, we further subdivided these genes into two groups: transport of mineral nutrients (72 genes) and secondary metabolites (187 genes). Compared to NM roots, 12 mineral transporters

were up- and 17 were down-regulated by both AM fungi (**Fig. 5A**). This suggests that a core set of mineral transporters in AM roots are similarly regulated independent of fungal benefits. However, a unique set of transporters were differentially regulated by each AM fungal species: 14 were up- and 13 were down-regulated in Ga165-colonized roots, while only 7 were up- and 12 were down-regulated in Ri09-colonized roots. From the identified 16 groups of mineral transporters (**Fig. 5B**), we focused on ammonium, nitrate, phosphate, and sulfate transporters with FPKM ≥ 10 , due to the key role that AM fungi play in the transport of these nutrients (**Fig. 5C-F**). Among the five differentially regulated ammonium transporters, one belongs to the AMT1 ammonium transporter family and four to the AMT2 family. Compared to AM roots, *AMT1;1* and two of the AMT2 family transporters (*AMT2;1* and *AMT2;6*) were highly expressed in NM roots, while *AMT2;3* and *AMT2;5* were specifically induced in mycorrhizal roots. *AMT2;3* was particularly up-regulated in Ri09-colonized roots (**Fig. 5C**).

We also identified seven differentially regulated nitrate transporters (**Fig. 5D**). Five belong to the nitrate/peptide transporter (NPF) family, and two are putative nitrate transporters. Interestingly, the most highly expressed nitrate transporter (*NPF4.12*) was highly upregulated in AM roots, particularly in Ri09-colonized roots. In contrast, five nitrate transporters were more highly expressed in Ga165-colonized roots than in Ri09-colonized roots (*NPF1.2*, *NPF1.5*, *NPF1.6*, *Medtr4g114340*, and *Medtr1g057460*), and two (*NPF1.2* and *Medtr1g057460*) also differed from control roots. Only the nitrate transporter *NPF5.24* and *Medtr1g057460* showed higher expression in NM roots than in either AM-colonized roots.

Seven phosphate transporters were differentially regulated (**Fig. 5E**). Four belong to the high-affinity inorganic phosphate transporter family PHT1 (*PT4*, *PT6*, *PT8*, and *PT9*), two were annotated as high-affinity inorganic phosphate transporters (*Medtr1g043290* and

Figure 5

Figure 5. Expression patterns of significantly differentially expressed mineral transporter genes in the roots of *Medicago truncatula* after colonization with different AM fungi. (A) Venn diagrams showing the number of mineral transporters that are significantly up- (yellow) or down-regulated (blue) in roots colonized by *Glomus aggregatum* 165 (Ga165) or *Rhizophagus irregularis* 09 (Ri09), or both. (B) Summary of the number of genes from specific gene annotation groups of mineral transporters that are up- or down-regulated in roots colonized by Ga165 or Ri09, or both (see also Supplemental File 5). Expression patterns of genes annotated as (C) ammonium, (D) nitrate, (E) phosphate, and (F) sulfate transporters in the roots of non-mycorrhizal plants (NM) (n=3) and plants inoculated with Ga165 (n=3) or Ri09 (n=4). The bars in C–F represent the mean of each treatment $\pm$ SEM. Expression levels are shown as fragments per kilobase of transcript per million mapped reads (FPKM), only genes with FPKM ≥ 10 are shown, significant differences (q-value < 0.05) in FPKM values between treatments were determined as part of the CuffDiff2 analysis and are indicated using different letters on the bars.

Medtr1g074930), and one as a phosphate transporter PHO1-like protein. As expected, two of the PHT1 transporter genes, *PT4* and *PT8*, were only expressed in AM roots, and *PT8* expression was higher in Ri09-colonized roots. By contrast, the expression of *Medtr1g074930* was strongly upregulated in Ga165-colonized roots compared to NM and Ri09-colonized roots. The PHO1-like transporter (*Medtr1g041695*) showed higher expression levels in NM than in AM roots.

We identified six sulfate transporters that were differentially regulated among the three treatments (**Fig. 5F**). Two were annotated as high affinity sulfate transporter 1 (SULTR1) family genes (*SULTR1.1* and *1.2*), three as sulfate/bicarbonate/oxalate exchanger and transporter genes (*SULTR2.2*, *3.1*, and *4.1*), and one as a sulfate transporter-like gene (*MOT1.1*). While the expression levels of *SULTR2.2* and *SULTR4.1* were higher in NM roots than in AM roots, the remaining sulfate transporters were upregulated by colonization with Ri09 (*SULTR1.1*), Ga165 (*SULTR3.1*), or by both fungi (*SULTR1.2* and *MOT1.1*).

In total, we identified 20 groups of secondary metabolite transporters in our dataset. Compared to NM control roots, 29 of these transporters were up- and 43 were downregulated in AM roots (**Fig. 6A**). Several secondary metabolite transporters were differentially regulated in Ga165 and Ri09-colonized roots. Among these, most belonged to six gene annotation groups, including ATP-binding cassette (ABC), amino acid, major facilitator superfamily (MFS), major intrinsic protein (MIP), peptide, and Sugars Will Eventually be Exported Transporters (SWEETs) (**Fig. 6B**). Here, we will only discuss those transporters that are known to play a role in AM symbiosis, or that were strongly differentially regulated by mycorrhizal colonization, but have not yet been functionally characterized.

Two white-brown-complex ABC transporter family proteins (*STR* and *STR2*; Zhang et al., 2010), three ABC transporter B family proteins (*AMN2*, *AMN3* [Roy, 2015], and *Medtr4g124040*),

and one drug resistance transporter-like ABC domain protein (*Medtr1g050525*) were strongly upregulated in AM roots, particularly in Ri09-colonized roots (**Fig. 6C**). The expression levels in Ga165-colonized roots were higher but did not always differ significantly from the NM roots.

Two amino acid transporters, *Medtr1g030660* and *Medtr2g101920*, and two peptide transporters, *PTR3* and *OPT15*, were upregulated in mycorrhizal roots (**Fig. 6D**). As expected, *NOPE1*, an N-acetylglucosamine transporter of the major facilitator superfamily (MFS) that is required for AM symbiosis (Nadal et al., 2017), was also highly upregulated in AM roots (**Fig. 6E**). Three major intrinsic protein (MIP) transporters, *NIP1;5*, *NIP1;6*, and *NIP3;1*, which are often classified as aquaporins, were all upregulated in mycorrhizal roots. *NIP1;5* was particularly highly expressed in Ri09-colonized roots, while *NIP1;6*, *NIP3;1* and *NIP6;2* showed higher expression levels in Ga165-colonized roots.

SWEETs are known to play a crucial role in AM symbiosis (Doidy et al., 2019), and five were differentially expressed in AM roots. *SWEET1.2* was upregulated by colonization with either fungus, but especially with Ri09. *SWEET3.3* and *SWEET12*, were only upregulated in Ri09-colonized roots, whereas *SWEET7* and *SWEET13* were only upregulated in Ga165-colonized roots (**Fig. 6F**). The variation in the expression patterns of these five *SWEET* genes suggests that sugar transport is strongly regulated in response to fungal benefit.

Photosynthesis-related genes in the shoots are upregulated in response to colonization with *Rhizophagus irregularis* 09

To identify gene groups that were differentially expressed in the shoots of mycorrhizal plants, we performed GO enrichment analysis on several gene clusters shown in **Fig. 2F**. Due to the low number of genes, the analysis could not be completed for clusters A and B; also, given the large

Figure 6

Figure 6. Expression patterns of significantly differentially expressed secondary metabolite transporters in the roots of *Medicago truncatula* after colonization with different AM fungi. (A) Venn diagrams showing the number of secondary metabolite transporters that are significantly up- (yellow) or down-regulated (blue) in roots colonized by *Glomus aggregatum* 165 (Ga165) or *Rhizophagus irregularis* 09 (Ri09), or both. (B) Summary of the number of genes from specific gene annotation groups of secondary metabolite transports that are up- or down-regulated in roots colonized by Ga165 or Ri09, or both (see Supplemental File 6). Expression patterns of genes annotated as (C) ATP-binding cassette (ABC), (D) amino acid or peptide, (E) major facilitator superfamily (MFS) or major intrinsic protein (MIP), and (F) sugar transporters in the roots of non-mycorrhizal plants (NM) (n = 3) and plants inoculated with Ga165 (n = 3) or Ri09 (n = 4). The bars in C-F represent the mean of each treatment $\pm$ SEM. Expression levels are shown as fragments per kilobase of transcript per million mapped reads (FPKM); only genes with FPKM ≥ 10 are shown; significant differences (q-value < 0.05) in FPKM values between treatments were determined as part of the CuffDiff2 analysis and are indicated using different letters on the bars. Abbreviations in B: G3P, glycerol-3-phosphate; NAT, nucleobase-ascorbate transporter; OST, organic solute transporter; TPT, triose-phosphate transporter; TRAPP, transport protein particle.

number of genes in cluster C, we divided the cluster into two groups dependent on whether the genes were upregulated by Ri09 (cluster C1, **Supplemental Fig. S9A**) or by Gal65 colonization (cluster C2, **Supplemental Fig. S9B**). In addition, we performed GO enrichment analysis with the cluster D genes which are downregulated in Ri09-colonized plants (**Supplemental Fig. S9C**).

The GO terms that were enriched in the shoots of Ri09-colonized plants were cellular components and biological processes associated with photosynthesis (**Supplemental Fig. S9A**). The five genes linked with these GO terms were annotated as follows: *Medtr1g115410*, photosystem II reaction center family protein (orthologous to PsbP in *Arabidopsis thaliana*); *Medtr2g082580*, oxygen-evolving enhancer protein (orthologous to PsbQ-like protein 2 in *A. thaliana*); *Medtr5g018670*, photosystem II oxygen-evolving enhancer protein; *Medtr1g015290*, ultraviolet-B-repressible protein (orthologous to PsbX in *A. thaliana*); and *Medtr1588s0010*, ATP synthase F1, gamma subunit. The expression levels of *PsbP*, *PsbQ-like protein 2*, *PsbX*, and the ATP synthase gene were significantly higher in the shoots of Ri09-colonized plants than in NM plants (**Fig. 7A**). Out of these genes, only PsbP was also significantly upregulated in Gal65-colonized plants. By contrast, *Medtr5g018670* was more highly expressed in the shoots of Gal65-colonized than in NM plants. Overall, these expression patterns imply that photosynthesis was more strongly induced in Ri09-colonized plants.

Biotic and abiotic stress-related genes are differentially regulated in the shoots of *Glomus aggregatum* and *Rhizophagus irregularis* colonized plants

Many of the GO terms that were enriched in the shoots of Gal65-colonized plants encompassed biological processes related to biotic stresses, including: “response to wounding” (GO:0009611), “defense response to fungus” (GO:0050832), and “defense response to bacterium” (GO:0042742)

(**Supplemental Fig. S9B**). The genes linked to these GO terms were annotated as: *Medtr4g054920*, cytochrome P450 family 94 protein; *Medtr4g120760*, pathogenesis-related protein bet V I family protein; *Medtr8g096900*, pathogenesis-related thaumatin family protein; *Medtr4g092010*, (3S)-linalool/(E)-nerolidol/(E,E)-geranyl linalool synthase; and *Medtr5g053950*, allene oxide cyclase. The fact that these GO terms were only enriched in Ga165-colonized plants suggests that the host plant responds to Ga165 as it would to a pathogen by upregulating defense responses (**Fig. 7B**).

In contrast, Ri09 colonization led to the down-regulation of genes (**Fig. 2F**, cluster D), associated with three GO terms: “response to hydrogen peroxide” (GO:0042542), “response to abiotic stimulus” (GO:0009628), and “response to bacterium” (GO:0009617) (**Supplemental Fig. S9C**). The genes included in these GO terms were annotated as: *Medtr1g048990*, superoxide dismutase; *Medtr7g069980*, ferritin; *Medtr8g059170*, NAC transcription factor-like protein; *Medtr7g079180*, late embryogenesis abundant protein; and *Medtr3g087590*, myo-inositol 1-phosphate synthase. The expression of *Medtr7g079180* was upregulated by both fungi, but the remaining abiotic stress response genes were more highly expressed in NM or Ga165-colonized plants than in Ri09-colonized plants (**Fig. 7C**). Collectively, the expression patterns of these abiotic stress response genes could indicate that Ri09 is more effective at conferring abiotic stress tolerance to *M. truncatula* than Ga165.

DISCUSSION

The growth and nutritional benefits of arbuscular mycorrhizal fungi are linked to mycorrhiza-induced transporter expression

Figure 7

Figure 7. Expression patterns of significantly differentially expressed genes in the shoots of *Medicago truncatula* colonized by different AM fungi. Expression levels of genes involved in (A) photosynthesis, (B) response to biotic stress, and (C) response to abiotic stress in non-mycorrhizal plants (NM; n=4) or plants colonized by *Glomus aggregatum* 165 (Ga165, n=4) or *Rhizophagus irregularis* 09 (Ri09, n=3). Bars represent the mean of each treatment $\pm$ SEM. Expression levels are shown as fragments per kilobase of transcript per million mapped reads (FPKM), and significant differences (q-value < 0.05) in FPKM values between treatments were determined as part of the CuffDiff2 analysis and are indicated using different letters above the bars (see also Supplemental Table S4).

When compatible host plants are inoculated with AM fungi, colonization most often leads to increased growth and nutrient uptake, especially in nutrient-deprived conditions (Chandrasekaran, 2020). We found that under N and P limitation, Ri09 promoted greater growth and provided more N and P to *M. truncatula* than Ga165 (**Fig. 1**). This observation is consistent with previous findings where we demonstrated that Ri09 is more cooperative than Ga165 (Kiers et al., 2011; Fellbaum et al., 2014; Wang et al., 2016) and with the expression patterns of mycorrhiza-induced N and P transporters. For example, the known AM-induced ammonium transporters *AMT2;3* and *AMT2;5* were upregulated by Ga165 and Ri09 (**Fig. 5C**). The expression levels of *AMT2;3* in AM roots were lower than that of *AMT2;5*, but *AMT2;3* expression was more strongly upregulated by Ri09 than by Ga165 colonization. We also found that *NIP1;5* is strongly AM-induced (**Fig. 6E**), and while its role in the AM symbiosis has not yet been characterized, the soybean ortholog *Nod26* plays a crucial role in ammonium transport in the rhizobia-legume symbiosis (Frare et al., 2018). Although the AM fungi were provided with $^{15}\text{NH}_4\text{Cl}$ as a N source, we also found that the nitrate transporter *NPF4.12* was strongly induced in AM roots, especially in Ri09-colonized roots (**Fig. 5D**). Aloui et al. (2018) found that *NPF4.12* is enriched in the proteome of mycorrhizal *M. truncatula* roots, and recently the rice ortholog *OsNPF4.5* was functionally characterized as an AM-induced nitrate transporter (Wang et al., 2020). The stronger expression of ammonium and nitrate transporters in Ri09 colonized roots aligns with the observed ^{15}N increase in the shoots of Ri09-colonized plants.

The AM-induced P transporters *PT4* and *PT8* were both highly expressed in AM colonized roots, especially Ri09-colonized roots (**Fig. 5E**). Similarly, colonization with both fungi, but particularly with Ri09, caused an increase in shoot P content. However, *Medtr1g074930*, a putative high affinity inorganic phosphate transporter, was strongly upregulated in Ga165-colonized roots

(**Fig. 5E**). This P transporter is upregulated during P deficiency (Wang et al., 2017); as such, the upregulation of *Medtr1g074930* in our study could indicate that colonization by Ga165 resulted in P deficiency in the host plant; but, this does not appear to be the case as *Medtr1g074930* was not upregulated in the roots of NM plants (**Fig. 5E**) and Ga165-colonized roots had a higher P concentration than NM roots (**Supplemental Fig. S3**). Regardless, others have similarly observed that the expression of high affinity P transporters are differentially regulated in the host by colonization with different species of AM fungi (Grunwald et al., 2009).

Like N and P, AM fungi also play a critical role in supplying sulfur to the host plant (Sieh et al., 2013). We observed an upregulation of two sulfate transporters, *SULTR1.1* and *SULTR1.2* (**Fig. 5F**). Both are upregulated in mycorrhizal *M. truncatula* roots, although not consistently (Casieri et al., 2012; Wipf et al., 2014). The *SULTR1.2* ortholog in *Lotus japonicus* appears to play an important role in both direct and mycorrhizal sulfur uptake pathways, and its promoter is active in arbuscule-containing cells (Giovannetti et al., 2014). We also observed that the putative sulfate transporter gene *MOT1.1* was upregulated in Ga165 and Ri09-colonized roots (**Fig. 5F**). However, the involvement of *MOT1.1* in sulfur transport has not been experimentally demonstrated yet, but it is phylogenetically related to *MOT1.3*, which is a molybdate transporter crucial for the rhizobia-legume symbiosis (Tejada-Jiménez et al., 2017).

Patterns of photosynthesis, carbon transporter, and lipid biosynthesis gene expression in the host plant are linked to the nutritional benefits provided by the fungal symbiont

In exchange for the nutritional benefits, plants transfer between 4 and 20% of their net fixed carbon in form of carbohydrates and lipids to their fungal symbiont (Wright et al., 1998; Roth and Paszkowski, 2017). However, both the nutritional benefits that AM fungi provide and the increased

carbon sink they create are known to stimulate an increase in photosynthesis (Kaschuk et al., 2009). In line with this, we observed that the expression levels of photosynthesis-related genes were more strongly induced in Ri09- than in Ga165-colonized plants (**Fig. 7A**); thus, the elevated growth response provided by Ri09 (**Fig. 1A-B**) was not only due to increased nutrient transfer, but also likely due to an increase in photosynthesis.

Our previous studies have shown that host plants preferentially allocate more carbon to the more cooperative AM fungus Ri09 (Kiers et al., 2011). Yet, we found that the putative lipid transporters *STR* and *STR2* were upregulated in AM roots, but did not differ significantly between Ga165 and Ri09-colonized roots (**Fig. 6C**). Nevertheless, we did observe that the expression levels of the lipid biosynthesis gene *RAM2* (**Fig. 4**) and the sugar transporter *SWEET1.2* (**Fig. 6F**) were higher in Ri09-colonized roots, which is suggestive of increased transport of carbon resources to the fungus in exchange for elevated nutritional benefits (Bravo et al., 2017; An et al., 2019).(An et al., 2019)

Although *SWEET1.2* is highly upregulated during AM colonization, particularly in arbuscule-containing cortical cells, it is not essential for the maintenance of the AM symbiosis, most likely because of functional redundancy between *SWEET1.2* and other *SWEETs* (An et al., 2019). We found that *SWEET3.3* and *SWEET12* were more strongly upregulated in Ri09-colonized roots (**Fig. 6F**); both are upregulated in arbuscule-containing cortical cells in *M. truncatula* (Sameeullah et al., 2016). Interestingly, *SWEET7* and *SWEET13* were more strongly induced in Ga165-colonized roots, but *SWEETs* perform diverse physiological functions, and their upregulation does not necessarily increase sugar transport to the fungus. For example, some *SWEET* transporters in *Arabidopsis* localize to the tonoplast and restrict sugar efflux from the root (Chen et al., 2015). Given the variable expression patterns of these five *SWEETs* in Ri09 and

Gal65-colonized roots and the varying roles they may play in sugar efflux and retention, functionally characterizing them will improve our understanding of the mechanisms controlling carbon allocation during AM symbiosis.

Arbuscule development is regulated by the host based on the nutritional benefit of the symbiont

Based on the elevated expression levels of known mycorrhiza-induced transporters that localize to the PAM (*PT8*, *AMT2;3*, and *SWEET1.2*) and of two genes involved in regulating arbuscular development and degeneration (*RADI* and *MYBI*) (**Fig. 4**), we hypothesized that these expression levels might be linked to increased arbuscule abundance and size in Ri09-colonized roots, which would increase the surface of the mycorrhizal interface. To test this, we conducted a short-term experiment and examined the number and average size of arbuscules in Ri09- and Gal65-colonized roots when arbuscules begin to form. Even in this short-term experiment, growth differences among the different treatments were already clearly visible (**Fig. 8A**). We found that Ri09 colonized the roots faster and formed more and larger arbuscules than Gal65 (**Fig. 8C-G; Supplemental Fig. S10**), suggesting a greater surface area of the PAM in Ri09 than in Gal65-colonized roots. A similar reduction in arbuscule size was observed previously in *mtpt4* mutants (Javot et al., 2007). Thus, our results strongly indicate that the host is rewarding Ri09 but likely sanctioning Gal65 based on the nutritional benefits that they provide.

Arbuscular mycorrhiza-induced changes in the expression of host abiotic and biotic stress response genes are species specific

Figure 8

Figure 8. Colonization patterns and arbuscule size of *Glomus aggregatum* 165 and *Rhizophagus irregularis* 09 in *Medicago truncatula* roots three weeks post-inoculation. (A) Image of non-mycorrhizal *M. truncatula* plants compared to either *G. aggregatum* 165 (Ga165) or *R. irregularis* 09 (Ri09) colonized plants. (B–D) Representative images of ink-stained NM roots (B), Ga165-colonized roots (C), and Ri09-colonized roots (D) (scale bar = 200 μm). (E–F) Representative confocal images of Ga165 (E) and Ri09-colonized (F) roots containing arbuscules and stained with wheat germ agglutinin Alexafluor-488 (scale bar = 50 μm). (G) Box and whisker plots embedded in violin plots displaying the size distribution for the width of all arbuscules in Ga165 (light grey) and Ri09 (dark grey) colonized roots. In 11 and 15 colonized roots, 132 and 80 arbuscules were evaluated for Ga165 and Ri09, respectively. The mean arbuscule width for each fungal species was compared using a student's t-test (**** $p < 0.0001$).

Although the primary focus of this study was to evaluate both resource exchange and transporter expression, the global gene expression data also provided some unique insights into biotic and abiotic stress responses. Mycorrhizal growth responses are context-dependent and fall along a mutualism to parasitism continuum (Johnson and Graham, 2013). In natural environments, plants are colonized by communities of AM fungi, which supports the idea of functional complementarity, where a host plant experiences a broad range of benefits that are provided by specific members of the AM fungal community (*e.g.*, enhanced nutrient availability or increased disease resistance; Jansa et al., 2008). While Ga165 does not seem to provide sufficient nutritional benefits to elicit a positive growth response, it is possible that like other AM fungal species it may provide resistance to one or more abiotic stresses (*e.g.*, salinity or heavy metal accumulation), none of which were evaluated in this study.

For example, Ga165 more strongly up-regulated defense-response genes in the shoots (**Fig. 7B**), two of which (*Medtr4g120760* and *Medtr8g096900*) are upregulated in alfalfa when challenged with the foliar fungal pathogen *Phoma medicaginis* (Li et al., 2019a) and pea aphids (*Acyrtosiphon pisum*; Li et al., 2019b). As such, Ga165 may be more effective than Ri09 at priming host defense responses thereby preventing infection from pathogens and mitigating arthropod attack as has been described previously for other AM fungal species (Liu et al., 2007; Sharma et al., 2017).

We also observed that genes associated with the GO term “response to water deprivation” (GO:0009414; 6 genes) were upregulated in Ga165-colonized roots (**Supplemental Fig. S7A**). Three of these genes — *Medtr5g063670*, *Medtr7g093170*, and *Medtr8g026960* — were very strongly upregulated and are annotated as annexin D8, seed maturation protein, and homeobox associated leucine zipper protein, respectively. Although the precise role of annexin D8 is not

known, other annexins are differentially regulated during various abiotic and biotic stresses, and some seem to play a role during AM symbiosis (Roux et al., 2012). The seed maturation protein *Medtr7g093170* has been characterized as the late embryogenesis abundant protein LEA1370, which is upregulated under various abiotic stresses, including abscisic acid treatment, cold, salinity and dehydration (Zhang et al., 2020). Finally, the homeobox associated leucine zipper protein encoded by *Medtr8g026960* was characterized as a transcription factor that is upregulated during salt stress in alfalfa (Postnikova et al., 2013). However, since these genes are not yet functionally characterized, the question whether Ga165 colonization could contribute to an alleviation of these abiotic stresses, or whether host plants respond similarly to abiotic stress and to Ga165 colonization, requires further studies.

It is also possible that Ga165 truly is a low-benefit symbiont capable of persisting in a community with high benefit AM fungi thereby avoiding both detection and subsequent sanctioning by the host plant for poor performance (Hart et al., 2013). Avoiding detection could be carried out using effectors, as has been described in the model AM fungus *R. irregularis* DAOM197198 that counteracts the plant immune program with the small, secreted peptide SP7 (Kloppholz et al., 2011). Indeed, in our root expression data we observed that 11 defense response genes, including three defensins (*Medtr8g012810*, *Medtr8g012815*, and *Medtr8g012845*) were strongly up-regulated in Ri09, but not in Ga165-colonized roots (**Supplemental Fig. S7B, Supplemental Table S5**). Defensins are known to inhibit pathogen growth (Maróti et al., 2015), so it is tempting to speculate that Ga165 could temper host defense responses to avoid detection and sanctioning. However, the defensin *DefMdl* when overexpressed upregulates the expression of the GRAS transcription factor *RAM1*, which plays a critical role in arbuscule branching (Park

et al., 2015; Uhe et al., 2018). *RAMI* was highly upregulated in Ga165 and Ri09 colonized roots suggesting that defensins might have a role in AM symbiosis.

Global analysis of transporter expression in *Medicago truncatula* roots revealed interesting candidates for future functional characterization

This study has provided novel insights into the physiological and transcriptomic response of a host plant to colonization by both high and low benefit AM fungi and **Fig. 9** provides an overview of these responses. The experimental approach allowed us to identify both the molecular mechanisms controlling the physiological responses we observed and transporters that are both commonly and uniquely regulated by high (Ri09) and low benefit (Ga165) AM fungal strains. While the primary focus of our study is on N, P, and carbon transporters in the roots of *M. truncatula* colonized by Ga165 or Ri09, our global analysis of all differentially expressed transporters uncovered some interesting gene candidates with unique expression patterns (**Figs. 5 and 6**). Although there was a core set of transporters similarly induced by both fungi, a smaller set were expressed differently, and many are prime candidates for future functional analysis.

Multiple ABC transporters were induced by AM colonization (**Fig. 6C**), and two of these, *STR* and *STR2*, have already been studied extensively (Zhang et al., 2010). Two others, *AMN2* and *AMN3*, are also well studied, but their functional role remains elusive (Roy, 2015). The two remaining genes, *Medtr4g124040* and *Medtr1g050525*, have not been functionally characterized and could represent interesting targets for future studies. The amino acid transporters *Medtr1g030660* and *Medtr2g101920* and the peptide transporters *PTR3* and *OPT15* were all upregulated in mycorrhizal roots. None of these have been functionally characterized, but the protein product of *Medtr2g101920* was isolated from the plasma membrane of AM roots (Aloui et

Figure 9

Figure 9. Summary of the physiological and transcriptomic responses in roots and shoots of *Medicago truncatula* colonized by either *Glomus aggregatum* 165 or *Rhizophagus irregularis* 09. Physiological data include the analysis of plant biomass and phosphorus and nitrogen-15 contents in plant tissues. Transcriptomic data include the expression patterns of nutrient transporters and enrichment of gene ontology terms. Yellow up-arrows indicate an increase, equal signs (=) indicate no change, and blue down-arrows indicate a decrease in each response compared to non-mycorrhizal plants. Arrow size indicates the degree of the response.

understanding how these mechanisms are regulated will likely allow for improved use of this symbiotic association in the sustainable production of food, feed, fiber, and fuel.

MATERIAL AND METHODS

Biological materials and growth conditions

M. truncatula ‘Jemalong A17’ seeds were acid scarified in 36N H₂SO₄ (Fisher Scientific Inc., Waltham, MA, USA), surface sterilized with 8.25% sodium hypochlorite (Clorox® bleach, The Clorox Company, Oakland, CA, USA), rinsed with sterile Type 1 water and imbibed at 4°C overnight. The seeds were then transferred onto moist autoclaved filter paper in Petri dishes and kept in the dark for 3 d. Finally, the Petri dishes were placed in ambient conditions for 7 d. Fully germinated seedlings were transferred into the root compartment (RC) of a custom-made, two-compartment pot measuring 12 cm x 8 cm x 8 cm (L x W x H; **Supplemental Fig. S1**). The RC was separated from the hyphal compartment (HC) by a 0.1 cm-thick plastic divider sealed on all sides by silicone (Aqueon, Franklin, WI, USA). A hole in the middle of the divider (~3.12 cm in diameter) was covered on both sides with a sheet of fine nylon mesh with 50 µm pores. Between these sheets was a coarse nylon mesh with 1000 µm pores to create an air gap and inhibit mass flow between the RC and the HC. This allowed fungal hyphae, but not roots, to crossover from the RC to the HC (**Supplemental Fig. S1**). Both compartments were filled with 250 ml of sterilized soil substrate containing 40% sand, 20% perlite, 20% vermiculite, and 20% soil by volume. The soil substrate had an original concentration of 4.81 mg kg⁻¹ of Olsen’s extractable phosphate, 10 mg kg⁻¹ of NH₄⁺, 34.4 mg kg⁻¹ of NO₃⁻, and a pH of 8.26. These nutrient levels were sufficiently low to induce host demand for N and P and to stimulate AM colonization.

Fungal inoculum for *Rhizophagus irregularis* isolate 09 (Ri09; collected from Southwest Spain by Mycovitro S.L. Biotechnología ecológica, Granada, Spain) and *Glomus aggregatum* isolate 165 (Ga165; collected from the Long Term Mycorrhizal Research Site, University of Guelph, Canada) were produced using axenic root organ cultures of Ri T-DNA transformed carrot (*Daucus carota* clone DCI) grown on minimal medium (St-Arnaud et al., 1996). After approximately eight weeks of growth, spores were isolated by blending the medium in 10 mM citrate buffer (pH 6.0). Then, at transplanting, each seedling was inoculated with ~0.4 g mycorrhizal roots and ~500 (long-term experiment) or ~350 (short-term experiment) spores of either Ri09 or Ga165. Non-mycorrhizal (NM) controls received a similar quantity of double autoclaved roots and spores. After transplanting and fungal inoculation, the plants were grown in a growth chamber (model TC30; Conviron, Winnipeg, MB, Canada) with a 25°C day/20°C night cycle, 30% relative humidity, and a photosynthetic photon flux of ~225 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Experimental design

Throughout the experiment, the position of the plants within the growth chamber was randomized three times. In the long-term experiment, 4 mM $^{15}\text{NH}_4\text{Cl}$ (Sigma Aldrich, St. Louis, USA) and 0.5 mM KH_2PO_4 in a modified Ingestad solution (Ingestad, 1960) were added to the HC of mycorrhizal and nonmycorrhizal plants five weeks post-inoculation. The plants were harvested two weeks later (seven weeks post-inoculation). For the short-term experiment, the plants were harvested three weeks post-inoculation to observe arbuscular development at an earlier time point.

Biomass determination, mycorrhizal quantification, and analysis of nitrogen and phosphorus contents in plants

At plant harvest, shoots and roots were separated to determine their respective fresh weight. Aliquots of both tissues were flash frozen in liquid N₂ and stored at -80°C for transcriptome analysis. An additional root aliquot was collected and stored in 50% ethanol at 4°C to assess AM colonization. The remaining plant tissue was dried at 70°C for 48 h and then shoot and root dry weights were recorded. To determine the level of AM colonization, root aliquots were cleared with 10% KOH at 80°C for 30 min, rinsed with tap water, and stained with 5% Sheaffer ink-vinegar (v:v) at 80°C for 15 min (Vierheilig et al., 1998). In both experiments, total mycorrhizal colonization rates were assessed using the gridline intersection method (McGonigle et al., 1990). In the short-term experiment, the same staining and counting methods were used in the short-term experiment, but we also specifically counted the number of arbuscules at root gridline intersections where AM fungi were present. In both experiments, we did not observe any fungal structures in the roots of NM control plants. Aliquots of dried shoot and root tissues were pulverized using a tissue homogenizer (Precellys 24 Dual, Cayman Chemical Company, Ann Arbor, MI, USA) and digested with 2N HCl for 2 h at 95°C. The P content in the plant tissues was spectrophotometrically determined at 436 nm after adding ammonium molybdate vanadate solution (Fisher Scientific, Pittsburgh, USA). Measurements of nitrogen-15 in plant tissues were performed by quantitative NMR spectroscopy as described previously (Kafle et al., 2018).

Fluorescent staining and confocal analysis of arbuscules

Ink-stained roots from the short-term experiment that contained arbuscules were washed with sterile 1X phosphate-buffered saline (PBS), incubated in 10% KOH for 2 d at room temperature, neutralized in 0.1 M HCl for 3 h at room temperature, and then rinsed in sterile PBS. The roots were then stained for confocal imaging as described in (Cope et al., 2019). Briefly, they were

incubated overnight at 4°C in PBS containing 2 $\mu\text{g ml}^{-1}$ Alexa Fluor 488TM conjugated to wheat germ agglutinin (WGA; Thermo Fischer Scientific, Waltham, MA, USA) and 10 $\mu\text{g ml}^{-1}$ propidium iodide. The next day, stained roots were mounted on a glass slide and immediately observed on an Olympus FV1200 confocal laser scanning microscope (Olympus, Shinjuku, Tokyo, Japan) with an Olympus UPlanApo 40 $\times$ /0.85 objective. The emission spectra of Alexa Fluor 488TM WGA (500 to 540 nm) and propidium iodide (580 to 630 nm) were detected after excitation with 488-nm and 561-nm lasers, respectively. Other settings used in the Fluoview v4.2c acquisition software (*e.g.*, laser intensity, gain, offset, magnification, airy units) were similar for all sample observations.

RNA isolation and sequencing

Root and shoot tissues from four biological replicates ($n = 4$) were homogenized in liquid N₂, and total RNA was extracted using the PureLinkTM RNA Mini Kit (Thermo Fisher Scientific). Extracted RNAs were treated with TURBOTM DNase (Thermo Fisher Scientific) and quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific). RNA quality was assessed using 2100 BioAnalyzer technology (Agilent Technologies). Approximately 100 ng of total RNA was used to construct poly(A) selection libraries using the Illumina TruSeq RNA Sample Preparation kit and 150-nucleotide single-end reads were sequenced using an Illumina NextSeq500 with high output flow cells (Genomics Sequencing Facility, South Dakota State University). The sequencing data were analyzed as described in the Supplemental Material and Methods.

Statistical analysis

Mean and standard error of the mean were determined from five independent biological replicates per treatment, unless mentioned otherwise. One-way ANOVA was used to identify statistically significant differences ($p \leq 0.05$) among all three treatments for dry weight, P concentration, P content, and nitrogen-15 enrichment. An LSD test was subsequently used for pairwise multiple comparisons to identify significant differences ($p \leq 0.05$) among treatments. These statistical analyses were conducted using Statistix 9 (analytical software, Tallahassee, FL, USA). For AM colonization and arbuscule size, statistically significant differences ($p \leq 0.05$) between the Ga165 and Ri09 mycorrhizal treatments were determined using a two-sample t-test in R v4.0.2 (The R Foundation, 2020). Only results that are significant at the $p \leq 0.05$ -level are primarily discussed. All F statistics and p-values are shown in **Supplemental Table S1**.

Supplemental Data

Supplemental Figure S1. Schematic model of the dual-compartment pot system used for this study.

Supplemental Figure S2. Percent colonization of *Medicago truncatula* roots inoculated with arbuscular mycorrhizal fungi.

Supplemental Figure S3. Shoot and root phosphorous concentration of *Medicago truncatula* colonized by arbuscular mycorrhizal fungi.

Supplemental Figure S4. Hierarchical clustering of biological replicates from root RNA sequencing data.

Supplemental Figure S5. Hierarchical clustering of biological replicates from shoot RNA sequencing data.

Supplemental Figure S6. Volcano plots of differentially expressed genes (DEGs) between treatments.

Supplemental Figure S7. Summary of gene ontology enrichment analysis of genes upregulated in the roots of *Medicago truncatula*.

Supplemental Figure S8. Summary of gene ontology enrichment analysis of genes downregulated in the roots of *Medicago truncatula*.

Supplemental Figure S9. Summary of gene ontology enrichment analysis of differentially expressed genes in the shoots of *Medicago truncatula*.

Supplemental Figure S10. Summary of arbuscular mycorrhizal colonization in *Medicago truncatula* at three-weeks post inoculation.

Supplemental Table S1. F statistics and p-values from one-way ANOVA for each experimental parameter.

Supplemental Table S2. Expression levels of genes associated with the strigolactone biosynthesis pathway and the common symbiosis signaling pathway in roots of *Medicago truncatula*.

Supplemental Table S3. Expression levels of genes downstream of the common symbiosis signaling pathway in roots of *Medicago truncatula*.

Supplemental Table S4. List of differentially regulated genes from shoot gene ontology enrichment analysis.

Supplemental Table S5. List of differentially regulated defense response genes from root gene ontology enrichment analysis.

Supplemental File 1. Significantly differentially expressed genes in roots

Supplemental File 2. Significantly differentially expressed genes in shoots

Supplemental File 3. Significant gene ontology terms in roots

Supplemental File 4. Significantly differentially expressed transporters in roots

Supplemental File 5. List of mineral transporters by type

Supplemental File 6. List of secondary metabolite transporters by type

Supplemental File 7. Significant gene ontology terms in shoots

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FIGURE LEGENDS

Figure 1. Biomass and nutrient content of *Medicago truncatula* plants colonized by different arbuscular mycorrhizal fungi. Shown are dry weight (A,B), phosphorus content (C,D), and % nitrogen-15 enrichment (E,F) of shoots (A,C,E) and roots (B,D,F). The plants were inoculated with either *Glomus aggregatum* 165 (Ga165) or *Rhizophagus irregularis* 09 (Ri09) and compared to non-mycorrhizal (NM) control plants. Bars represent the mean of each treatment ($n = 5$) $\pm$ SEM. Different letters on the bars indicate statistically significant ($p \leq 0.05$) differences based on one-way ANOVA and LSD test. F statistics and p-values are shown in Supplemental Table S1.

Figure 2. Significantly differentially expressed genes in roots (A–C) and shoots (D–F) of *Medicago truncatula* colonized by different AM fungi. (A, D) Number of significantly differentially expressed genes (DEGs; $\log_2[\text{fold-change}] > 2$ and a q-value < 0.05) in plants

colonized by *Glomus aggregatum* 165 (Ga165) or *Rhizophagus irregularis* 09 (Ri09) compared either to non-mycorrhizal (NM) control plants or to each other. (B, E) Venn diagrams of significantly upregulated (yellow) and downregulated (blue) DEGs unique to Ga165 and Ri09-colonized plants or shared by both. (C, F) Heat maps of fragments per kilobase of transcript per million mapped reads (FPKM) values + 1 for all significant DEGs from each treatment. Gene clusters are assigned based on second-order branching. Upregulated genes are shown in yellow and downregulated genes in blue with intensity based on row z-score. All DEGs in each plot for roots and shoots are listed in Supplemental Files 1 and 2, respectively.

Figure 3. Expression patterns of genes in the strigolactone biosynthesis pathway. (A) Diagram of the strigolactone biosynthesis pathway and the relative expression levels of genes in *Medicago truncatula* that encode components of the pathway shown as fragments per kilobase of transcript per million mapped reads (FPKM; blue=low, yellow=high) in roots from non-mycorrhizal (NM) plants or plants colonized by either *Glomus aggregatum* 165 (Ga165) or *Rhizophagus irregularis* 09 (Ri09). The expression patterns (FPKM) for four specific genes upregulated by Ri09-colonization are also shown, including (B) *More Axillary Growth 1a* (MAX1a), (C) *Carotenoid Cleavage Dioxygenase 7* (CCD7), (D) *Nodulation Signaling Pathway 1* (NSP1) and (E) NSP2 in NM roots (n=3) and roots inoculated with Ga165 (n=3) or Ri09 (n=4). Bars represent the mean of each treatment $\pm$ SEM. Significant differences (q-value < 0.05) in FPKM values between treatments were determined as part of the CuffDiff2 analysis and are indicated using different letters (see **Supplemental Table S2**). Abbreviations in A: GAP, glyceraldehyde 3-phosphate; DXS2, 1-deoxyxylulose 5-phosphate synthase2; MEP, 2-methyl-erythritol-4-phosphate; GGDP geranylgeranyl pyrophosphate; D27, Dwarf27; and PDR1a, pleiotropic drug resistance 1a.

Figure 4. Relative expression of six genes in the roots of *Medicago truncatula* after colonization with different AM fungi. Expression of (A) *Required for Arbuscule Development 1* (*RAD1*), (B) *MYB-like Transcription Factor 1* (*MYB1*), (C) *Phosphate Transporter 8* (*PT8*), (D) *Ammonium Transporter 2;3* (*AMT2;3*), (E) *Reduced Arbuscular Mycorrhization 2* (*RAM2*), and (F) *Sugars Will Eventually Be Exported Transporter 1.2* (*SWEET1.2*) are shown for non-mycorrhizal plants (NM, n=3) and plants inoculated with *Glomus aggregatum* 165 (Ga165, n=3) or *Rhizophagus irregularis* 09 (Ri09, n=4). Expression levels are shown as fragments per kilobase of transcript per million mapped reads (FPKM). Bars represent the mean of each treatment $\pm$ SEM. Significant differences (q-value < 0.05) in FPKM values between treatments were determined as part of the CuffDiff2 analysis and are indicated using different letters (see Supplemental Table S3).

Figure 5. Expression patterns of significantly differentially expressed mineral transporter genes in the roots of *Medicago truncatula* after colonization with different AM fungi. (A) Venn diagrams showing the number of mineral transporters that are significantly up- (yellow) or down-regulated (blue) in roots colonized by *Glomus aggregatum* 165 (Ga165) or *Rhizophagus irregularis* 09 (Ri09), or both. (B) Summary of the number of genes from specific gene annotation groups of mineral transporters that are up- or down-regulated in roots colonized by Ga165 or Ri09, or both (see also Supplemental File 5). Expression patterns of genes annotated as (C) ammonium, (D) nitrate, (E) phosphate, and (F) sulfate transporters in the roots of non-mycorrhizal plants (NM) (n=3) and plants inoculated with Ga165 (n=3) or Ri09 (n=4). The bars in C–F represent the mean of each treatment $\pm$ SEM. Expression levels are shown as fragments per kilobase of transcript per million mapped reads (FPKM), only genes with FPKM $\geq$ 10 are shown, significant differences (q-

value < 0.05) in FPKM values between treatments were determined as part of the CuffDiff2 analysis and are indicated using different letters on the bars.

Figure 6. Expression patterns of significantly differentially expressed secondary metabolite transporters in the roots of *Medicago truncatula* after colonization with different AM fungi. (A) Venn diagrams showing the number of secondary metabolite transporters that are significantly up- (yellow) or down-regulated (blue) in roots colonized by *Glomus aggregatum* 165 (Ga165) or *Rhizophagus irregularis* 09 (Ri09), or both. (B) Summary of the number of genes from specific gene annotation groups of secondary metabolite transports that are up- or down-regulated in roots colonized by Ga165 or Ri09, or both (see Supplemental File 6). Expression patterns of genes annotated as (C) ATP-binding cassette (ABC), (D) amino acid or peptide, (E) major facilitator superfamily (MFS) or major intrinsic protein (MIP), and (F) sugar transporters in the roots of non-mycorrhizal plants (NM) ($n = 3$) and plants inoculated with Ga165 ($n = 3$) or Ri09 ($n = 4$). The bars in C-F represent the mean of each treatment $\pm$ SEM. Expression levels are shown as fragments per kilobase of transcript per million mapped reads (FPKM); only genes with FPKM ≥ 10 are shown; significant differences (q -value < 0.05) in FPKM values between treatments were determined as part of the CuffDiff2 analysis and are indicated using different letters on the bars. Abbreviations in B: G3P, glycerol-3-phosphate; NAT, nucleobase-ascorbate transporter; OST, organic solute transporter; TPT, triose-phosphate transporter; TRAPP, transport protein particle.

Figure 7. Expression patterns of significantly differentially expressed genes in the shoots of *Medicago truncatula* colonized by different AM fungi. Expression levels of genes involved in (A) photosynthesis, (B) response to biotic stress, and (C) response to abiotic stress in non-mycorrhizal

plants (NM; n=4) or plants colonized by *Glomus aggregatum* 165 (Ga165, n=4) or *Rhizophagus irregularis* 09 (Ri09, n=3). Bars represent the mean of each treatment $\pm$ SEM. Expression levels are shown as fragments per kilobase of transcript per million mapped reads (FPKM), and significant differences (q-value < 0.05) in FPKM values between treatments were determined as part of the CuffDiff2 analysis and are indicated using different letters above the bars (see also Supplemental Table S4).

Figure 8. Colonization patterns and arbuscule size of *Glomus aggregatum* 165 and *Rhizophagus irregularis* 09 in *Medicago truncatula* roots three weeks post-inoculation. (A) Image of non-mycorrhizal *M. truncatula* plants compared to either *G. aggregatum* 165 (Ga165) or *R. irregularis* 09 (Ri09) colonized plants. (B–D) Representative images of ink-stained NM roots (B), Ga165-colonized roots (C), and Ri09-colonized roots (D) (scale bar = 200 μ m). (E–F) Representative confocal images of Ga165 (E) and Ri09-colonized (F) roots containing arbuscules and stained with wheat germ agglutinin Alexafluor-488 (scale bar = 50 μ m). (G) Box and whisker plots embedded in violin plots displaying the size distribution for the width of all arbuscules in Ga165 (light grey) and Ri09 (dark grey) colonized roots. In 11 and 15 colonized roots, 132 and 80 arbuscules were evaluated for Ga165 and Ri09, respectively. The mean arbuscule width for each fungal species was compared using a student's t-test (****p < 0.0001).

Figure 9. Summary of the physiological and transcriptomic responses in roots and shoots of *Medicago truncatula* colonized by either *Glomus aggregatum* 165 or *Rhizophagus irregularis* 09. Physiological data include the analysis of plant biomass and phosphorus and nitrogen-15 contents in plant tissues. Transcriptomic data include the expression patterns of nutrient transporters and

enrichment of gene ontology terms. Yellow up-arrows indicate an increase, equal signs (=) indicate no change, and blue down-arrows indicate a decrease in each response compared to non-mycorrhizal plants. Arrow size indicates the degree of the response.

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