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# Multiple mutualist effects on genomewide expression in the tripartite association between *Medicago truncatula*, nitrogen-fixing bacteria and mycorrhizal fungi

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## Abstract

**While all species interact with multiple mutualists, the fitness consequences and molecular mechanisms underlying these interactions remain largely unknown. We combined factorial ecological experiments with genomewide expression analyses to examine the phenotypic and transcriptomic responses of model legume *Medicago truncatula* to rhizobia and mycorrhizal fungi. We found synergistic effects of these mutualists on plant performance and examined unique features of plant gene expression responses to multiple mutualists. There were genomewide signatures of mutualists and multiple mutualists on expression, with partners often affecting unique sets of genes. Mycorrhizal fungi had stronger effects on plant expression than rhizobia, with 70% of differentially expressed genes affected by fungi. Fungal and bacterial mutualists had joint effects on 10% of differentially expressed genes, including unexpected, nonadditive effects on some genes with important functions such as nutrient metabolism. For a subset of genes, interacting with multiple mutualists even led to *reversals* in the direction of expression (shifts from up to downregulation) compared to interacting with single mutualists. Rhizobia also affected the expression of several mycorrhizal genes, including those involved in nutrient transfer to host plants, indicating that partner species can also impact each other's molecular phenotypes. Collectively, these data illustrate the diverse molecular mechanisms and transcriptional responses associated with the synergistic benefits of multiple mutualists.**

*Keywords*: legume, MME, rhizobia, RNA-Seq, symbiosis, transcriptome

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## Introduction

Mutualisms, interspecific interactions in which all participants benefit from the association, are extremely common in nature (Bascompte & Jordano 2006; Guimarães *et al*. 2006; Gustafson & Casper 2006; Palmer *et al*. 2010; Charlton *et al*. 2014; Godschalx *et al*. 2015), so much so that most organisms interact with many different mutualists simultaneously or sequentially throughout their lives. Empirical studies have recognized that complementarity and conflict among multiple mutualist can have substantial and diverse effects on the success of organisms that cannot be detected based on pairwise studies (Champawat 1990; Lau & Galloway 2004; Ness 2006; Bracken *et al*. 2007; Palmer *et al*. 2010; Vivarelli *et al*. 2011; McKeon *et al*. 2012), and theory predicting when we expect to see different outcomes is under development (Afkhami *et al*. 2014). However, despite this progress, we still have little knowledge of the performance outcomes of organisms balancing multiple, functionally distinct partner species or the molecular pathways influencing those outcomes. Here, we combine manipulative experiments and genomewide expression analysis to examine phenotypic and transcriptomic responses of the legume *Medicago truncatula* to its interaction with rhizobia and mycorrhizal fungi.

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Understanding the fitness consequences underlying multispecies mutualisms is important for evaluating the cascading effects mutualisms have on many ecological and evolutionary processes, such as succession (Rudgers *et al.* 2007), community assembly (Fontaine *et al.* 2006; Keller 2014a) and diversification (Joy 2013; Weber & Agrawal 2014). The nature of more complex multispecies associations is often not well understood (Stanton 2003; Afkhami *et al.* 2014), in contrast to our understanding of the biology of pairwise mutualisms. Multiple mutualists can vary along a continuum from those that confer similar rewards (*e.g.* multiple pollinator species; Lau & Galloway 2004; Bascompte & Jordano 2006) to those that confer functionally distinct rewards (*e.g.* a pollinator and mycorrhizal fungi that increase access to water and nutrition; Gange & Smith 2005; Cahill *et al.* 2008). While the effect of these mutualistic assemblages for an organism's performance can be difficult to predict, theoretical and empirical evidence suggests that multiple mutualists are more likely to have synergistic effects on performance when partner species provide functionally distinct and complementary benefits (Stachowicz & Whitlatch 2005; Palmer *et al.* 2010). Similarly, partners who receive the same reward from their shared mutualist may compete in such a way that leads to cascading negative effects on the shared mutualist (Ness 2006; Afkhami *et al.* 2014).

In terrestrial ecosystems, one ubiquitous type of multispecies mutualism is the tripartite interaction between plants, mycorrhizal fungi and nitrogen-fixing bacteria. An estimated 80% of land plants interact with mycorrhizal fungi, which can provide benefits like enhanced phosphorus and water acquisition to their host plants (Wang & Qiu 2006). Similarly, many legumes associate with rhizobia and other bacteria that fix atmospheric nitrogen; recent work has extrapolated that up to 90% of legumes—the third largest family of flowering plants—may participate in this type of interaction (M.E. Afkhami, D.L. Mahler, J.H. Burns *et al.*, unpublished manuscript). By conferring complementary benefits in nutrient-poor environments, these microbial symbionts can have synergistic effects on their host plant's performance as well as positive effects on one another (Larimer *et al.* 2014; Ossler *et al.* 2015). While meta-analysis has shown that the effects of growing plants with both rhizobia and mycorrhizal fungi are often positive, there is evidence for context dependency and, under some conditions, a cost of having both partners (Bethlenfalvay *et al.* 1982; Larimer *et al.* 2010). Cases of these microbes negatively affecting host plant performance could result from both symbionts competing for the same primary reward, photosynthetic carbon, and suggest that selection for host mechanisms to regulate these complex associations could exist.

The molecular basis of these plant–microbe interactions has been intensively explored from a pairwise perspective. Transcriptomic studies of plants grown with a single microbial mutualist, either rhizobia or mycorrhizal fungi, have begun to characterize the repertoire of symbiont-induced plant genes and highlight the molecular processes by which these partners interact (Hohnjec *et al.* 2005; Boscari *et al.* 2013). For example, mycorrhizal fungi induce genes that function in building cell walls, metabolism and transport (Hohnjec *et al.* 2005). Some prior research has elucidated genes that act in both symbioses, such as those shared in the 'common symbiosis pathway' (Markmann & Parniske 2009; Oldroyd *et al.* 2009), and studies comparing sets of genes induced or suppressed by each partner separately have provided further insight into the similarities and differences in how these symbioses affect plant gene expression in isolation (Salzer *et al.* 2000; Manthey & Krajinski 2004; Deguchi *et al.* 2007; Tromas *et al.* 2012). Moving beyond pairwise studies is necessary, however, because plants often associate with both of these microbial symbionts, and factorial experiments are required for identifying genes or pathways involved in either synergistic benefits or conflicts plants face when interacting with multiple partners. For example, while synergistic benefits may be expected ecologically (*e.g.* when partners confer complementary rewards), it is unclear whether this is due to upregulation of the same pathways, or the expression of novel genes and pathways when exposed to multiple mutualists (Fig. 1a). In contrast, if the same genes or pathways increase expression in response to one mutualist, but decrease expression in response to a second (Fig. 1b), there may be conflicting influences on expression level when plants associate with both partners, with unclear consequences for fitness. Transcriptomic studies can reveal mixtures of synergy and conflict in the expression of individual genes and pathways that would be inaccessible in studies focusing solely on whole-plant, composite phenotypes.

There are at least three, nonexclusive ways that multiple mutualists could affect transcription in host plants (Fig. 1c). First, it is possible that mutualists conferring different types of rewards may primarily affect different biochemical pathways, and consequently, they may predominately affect expression in unique sets of genes. Second, mutualists could have joint effects on the same genes, acting additively to impact expression of a focal gene or pathways. Third, multiple mutualists may cause interactive or nonadditive changes in expression that can only be detected from factorial experiments. For example, complementary rewards provided by symbionts could lead to changes in plant condition or vigour that in turn lead to nonadditive effects on gene

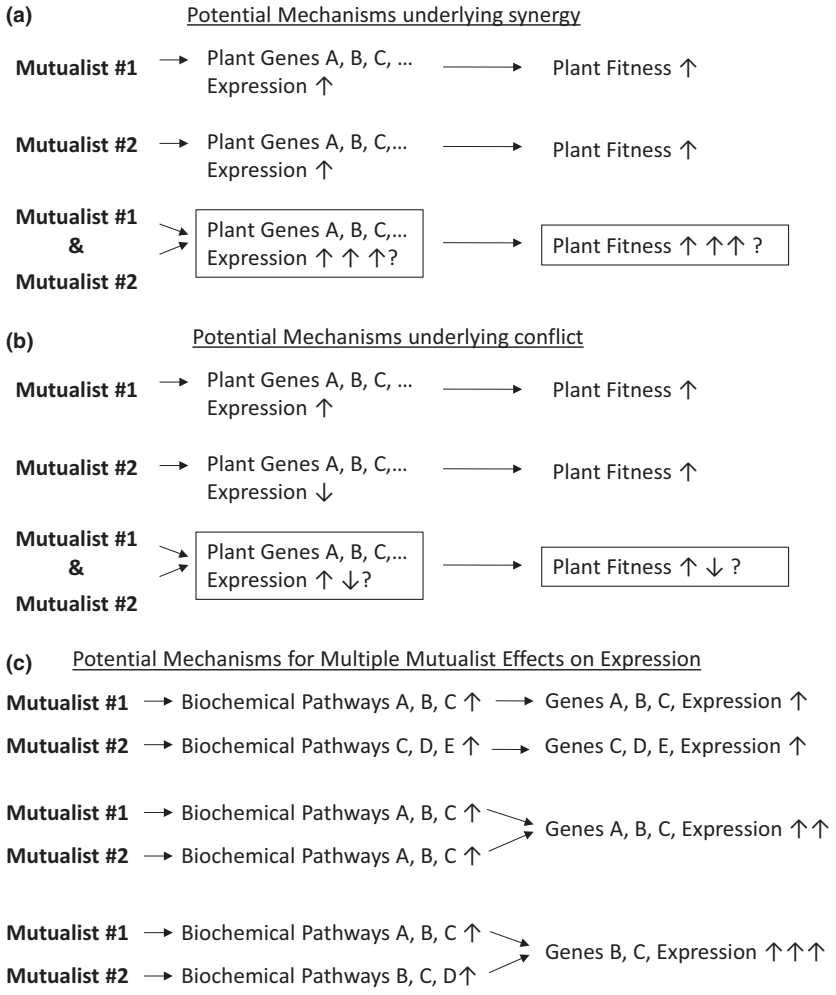


**Fig. 1** Schematic illustrating the potential synergistic or conflicting effects multiple mutualists can have on expression and plant fitness. (a) In individual pairwise experiments, mutualists 1 and 2 increase the expression of genes A, B and C and plant fitness compared to a no mutualist control (not shown). When grown together, multiple mutualists can lead to nonadditive improvements in plant fitness (indicated by ↑↑↑). It is unknown whether these nonadditive increases in plant fitness are associated with nonadditive increases in expression of genes A, B and C (↑↑↑?). (b) In individual pairwise experiments, mutualists 1 and 2 have opposing effects on the expression of genes A, B and C (indicated by ↑ or ↓), even though both improve plant fitness (↑). When grown together, it is unknown whether these genes show increased or decreased expression (↑↓?) and whether plant fitness increases or decreases (↑↓?) relative to a single-mutualist condition. (c) Schematic illustrating the diverse effects multiple mutualists could have on expression. In the first case, each mutualist stimulates separate pathways, with corresponding expression effects on unique genes. In the second case, the mutualists activate the same biochemical pathways and expression of the same genes in an additive manner. In the third case, the mutualists activate slightly nonoverlapping pathways, and the genes whose expression is affected by both mutualists show nonadditive expression.

expression and/or shifts in gene expression. Likewise, molecular regulation by host plants that reduces conflict and promotes cooperation among symbionts could lead to nonadditive effects on expression. Determining the prevalence of these alternatives will not only improve our understanding of molecular phenotypes associated with interacting with multiple mutualists, but also facilitate identifying pathways that could be the site of synergistic benefits or constraints plants face in evolving with multiple interspecific partners.

In this study, we took an RNAseq transcriptomic approach in the tripartite mutualism between the model legume *Medicago truncatula,* rhizobia and mycorrhizal fungi to address four fundamental questions about how organisms respond to multiple mutualist at a molecular level that cannot be addressed from bipartite studies. Specifically, we asked the following: (i) Do multiple mutualists have synergistic effects on plant performance and pervasive genomewide effects of multiple mutualists on expression (*i.e.* extensive effects on expression of

many genes throughout the genome)? (ii) Do microbial mutualists predominantly impact gene expression individually, additively or nonadditively? and (iii) Does one microbial partner have a stronger effect on gene expression than the other? Further, because these microbial partners are known to impact each other's performance under some conditions (Champawat 1990), we also examined the consequences of multispecies mutualisms for expression across the mycorrhizal genome, asking (iv): Does multispecies mutualism affect gene expression of one of the microbial partner?

## Methods

### *Study system*

We investigated the impact of multiple mutualist effects using the tripartite mutualism between *Medicago truncatula* ('Barrel Medic') and two microbial symbionts —rhizobia and mycorrhizal fungi—that differ substantially in the primary rewards they confer: phosphorus from fungi and fixed nitrogen from rhizobia (Larimer *et al.* 2014). *Medicago truncatula* ('Jemalong A17'), an annual Mediterranean-native plant, is a primary genomic model system for legumes (Young & Udvardi 2009). We used rhizobia (*Ensifer meliloti* Rm1021) and mycorrhizal fungi (*Rhizophagus irregularis* DAOM197198) that are known to occur with *M. truncatula* alone and in combination to expose plants to realistic microbial interactions they could face in nature (Zribi *et al.* 2004).

### *Experimental design*

We mechanically scarified seeds of *M. truncatula* JA17 (SARDI, Adelaide, South Australia), surface-sterilized them in a bleach solution, rinsed with sterile water and germinated them on sterile 0.8% water agar plates in the dark at 4 °C for 36 h (Garcia *et al.* 2006). After an additional 18 h at 22 °C, we planted germinants into pots in the glasshouse with no supplemental light or nutrients. Pots were constructed from nested magenta boxes with the lower box containing sterile DI water and a wick that transferred moisture to the upper box which contained sand as in Heath *et al.* (2010). To create a closed and sterile system, we sterilized the entire planting apparatus in an autoclave at 121 °C three times prior to use in the experiment and attached a sterile plastic bag to the top.

We planted 120 germinants in a completely randomized block design with a factorial manipulation of the presence of mycorrhizal fungi (M±) and rhizobia (R±) simultaneously. A quarter of the germinants from each of the five spatial blocks were left sterile (M−R−), a quarter were inoculated with rhizobia (M−R+), another quarter inoculated with mycorrhizal fungi (M+R−) and the final quarter with both microbes (R+M+). Mortality occurred immediately after planting and was rare (two plants); these germinants were replaced prior to treatment inoculation. We inoculated 1 week after planting and again 4 days later to ensure an opportunity for nodulation and colonization. Prior to inoculation, we grew rhizobia in TY media for 36 h and diluted to ~$10^6$ cells/ml ($OD_{600}$ = 0.1) with $ddH_2O$ as in (Simonsen & Stinchcombe 2014). We applied 1 ml of inoculant to each R+ germinants while R− germinants received the same 'inoculant' *without* rhizobia. We inoculated each M+ germinants with sterile water containing ~300 spores (Premier Tech, Rivière-du-Loup, Quebec, Canada; Antunes *et al.* 2008; Powell *et al.* 2009). M− germinants received the same solution after autoclaving four times (121 °C; 45 min cycle) to ensure spore inviability.

### *Harvest and phenotype data*

Two weeks postinoculation, we began collecting weekly leaf counts and branching data for each plant. We harvested plants after 7 weeks of growth, a time point selected to ensure microbial associations had developed but before senescence. Under sterile conditions, we flash-frozen roots from 60 plants in liquid nitrogen for RNA extraction—eight plants per microbial treatment for sequencing RNA with seven per treatment in reserve. We randomly sampled 1–2 plants per treatment from each of the five spatial blocks for RNAseq. We additionally harvested aboveground tissue from all plants and belowground tissue from the remaining 60 plants, then dried at 60 °C and weighed the biomass. We counted nodules on the roots of these plants and weighed five nodules per plant on a microbalance.

### *RNA extraction and sequencing*

We extracted RNA from the complete root system of 32 plants (eight per treatment combination) using Norgen Biotek's Plant/Fungi Total RNA Purification Kit (Sigma, www.sigmaaldrich.com) and then confirmed the quantity and integrity of the RNA on a Nanodrop 1000 (Thermo Scientific; www.thermofisher.com) and a 2100 Bioanalyzer (Agilent; www.agilent.com). All samples had high RNA Integrity Numbers (RINs = 9.18 ± 0.06 of 10), total RNA (6487 ± 482 ng) and RNA concentrations (132.38 + 9.84 ng/μl). The cDNA libraries were prepared using the TruSeq Stranded mRNA Sample Preparation Kit and sequenced on the Illumina HiSeq 2000 platform (100-bp paired-end reads) with 8 samples per lane (Genome Quebec at McGill University).

### *Performance analysis*

We analysed leaf and branching data collected weekly with SAS (2015) using a repeated-measures ANOVA that included block (*i.e.* to account for spatial variation within the glasshouse), rhizobia (presence/absence), mycorrhizal fungi (presence/absence) and week of measurement, as well as two-way and three-way interactions between bacterial treatment, fungal treatment and week. For measurements of plant above- and belowground biomass and rhizobia nodule counts, we used an ANOVA with fixed factors of rhizobia and fungi treatments and their interaction as well as a block effect. Nodule mass was analysed in the same way except we excluded R− plants and removed the rhizobia factor and its interaction. As a coarse indicator of mycorrhizal abundance and performance, we analysed the percentage of reads that mapped to the mycorrhizal genome (natural log-transformed to improve normality) using an ANOVA with factors of rhizobia and fungi treatments and their interaction.

### *Read mapping and differential gene expression analysis*

We used TopHat v2.0.12 with Bowtie v2.2.3 (Trapnell *et al.* 2009; Langmead & Salzberg 2011) to map transcripts to the *M. truncatula* JA17 (Mt4.0; www.medicagohapmap.org) and *R. irregularis* DAOM 197198 (v1.0; genome.jgi-psf.org/Gloin1/Gloin1.home.html) reference genomes. Tophat was run allowing a maximum of two mismatches per read, and annotation files for both genomes were provided to guide identification of splice junctions. For both plant and fungi, we determined the number of reads per gene using Htseq v1.8.1 (Anders *et al.* 2014) supplied with annotation files. Deseq2 was then used to test for genomewide effects (i.e. effects on many genes, throughout the genome) of multiple mutualists on expression using principal components and differential expression analyses (R package 2013; Love *et al.* 2014). To analyse differential expression of plant genes, we fit a model with fixed effects of the presence/absence of mycorrhizal fungi and of rhizobia, as well as their interaction. We tested for significant main effects of the microbial treatments (*i.e.* whether expression of each plant gene is impacted by rhizobia and/or mycorrhizal fungi) as well as their interaction using likelihood ratio tests. As described in Table 1, expression of genes may be affected by these mutualists in three ways: (i) *individually/uniquely*—only rhizobia *or* mycorrhizal fungi impacts expression, which is indicated by a significant main effect of either rhizobia *or* mycorrhizal fungi, (ii) *additively*—both mutualists impact expression but the effects are independent (*i.e.* effects with single partners can be summed to calculate effects with multiple partners) which is indicated by a significant main effects of both mutualists but no significant interaction and (iii) *nonadditively*—both mutualists impact expression, and effects of one mutualist is impacted by the presence of the other (*i.e.* effects with single partners *cannot* be summed to calculate effects with multiple partners) which is indicated by a significant interaction term. We repeated this analysis to examine differential expression of fungal genes but only included fungi-inoculated plants and fit a model with a fixed effect of rhizobia (presence/absence). To account for multiple comparisons for plant and mycorrhizal genes, we used Benjamini–Hochberg adjusted P-values of <0.05 as calculated in Deseq2.

**Table 1** Effects of multiple mutualists on gene expression

| | | Statistical determination** | | | Experimental Results*** | |
|---|---|---|---|---|---|---|
| Expression category | Change in expression formula* | R main effect | M main effect | R*M interaction | # Genes in category | Example in Fig. 3 |
| *Unique/independent effects* | | | | | | |
| Rhizobia (R) only | $\Delta RM = \Delta R$ | * | — | — | 2028 | — |
| Mycorrhizal fungi (M) only | $\Delta RM = \Delta M$ | — | * | — | 4131 | — |
| *Joint effects* | | | | | | |
| Additive | $\Delta RM = \Delta R + \Delta M$ | * | * | — | 561 | A |
| Non-additive | $\Delta RM \neq \Delta R + \Delta M$ | *? | *? | * | 62 | B-D |

*Relationship between the change in expression associated with the presence of both symbionts (ΔRM) and change in expression associated with the presence of rhizobia only (ΔR) and/or mycorrhizal fungi only (ΔM).

**Statistical expectations for each expression category where * indicates that it must be a significant effect, *? indicates that it may or may not be significant effect, and—indicates nonsignificant. Please note that 'additive' must have significant main effects of both R and M, but not a significant interaction. 'Nonadditive' must have a significant interaction (and may or may not have significant main effects).

***Results from differential expression analysis, including the number of genes we detected in each category and reference to relevant panels from Fig. 3 with example graphs.

We used agriGo (Du *et al.* 2010), which natively supports the *M. truncatula* v4.0 genome, to detect enrichment of gene functions within differentially expressed genes with the Yekutieli false discovery rate adjustment to determine significance accounting for multiple tests. The enrichment analyses identify overrepresented gene functions that are associated with the presence of rhizobia, mycorrhizal fungi or their interaction. These functions are broken into three main groupings of Gene Ontology (GO) functions—cellular components, molecular functions and biological processes—with many nested levels of increasing specificity within each of the main groupings (*e.g.* catalysis activity within molecular function, hydrolase activity within catalysis activity and beta-alanyl-dopamine hydrolase activity within hydrolase activity). For these analyses, we required a minimum of five mapping entries for tests done in the main categories (*i.e.* genes affected by only one symbiont, genes affected additively by both partners or genes affected nonadditively) and a minimum of two mapping entries for follow-up tests (*e.g.* see below). To investigate whether one partner was more important for determining expression when plants participate in multispecies mutualism, we evaluated for each gene whether its change in expression with both partners relative to the control [(M+R+)–(M−R−)] was more similar to the change in expression resulting from association with just rhizobia [(M+R+)–(M−R+)] or just fungi [(M+R+)–(M+R−)]. We then used a chi-squared test to determine whether the number of genes in these two categories deviated significantly from the null expectation that half of the genes would have changes in expression closer to fungi and half closer to rhizobia.

## Results

### *Do multiple mutualists have synergistic effects on plant performance?*

Multiple mutualists had synergistic effects on *M. truncatula* performance with M+R+ plants having significantly more trifoliate leaves (Fig. 2A, Table S1, Supporting information; fungi × rhizobia interaction: $F_{1,339}$=10.69, P = 0.0012), higher above-ground biomass (Fig. 2B, Table S1, Supporting information; fungi × rhizobia interaction: $F_{1,111} = 5.16$, $P = 0.0250$) and more branches (Fig. S1A, Table S1, Supporting information; fungi × rhizobia: $F_{1,339} = 8.74$, $P = 0.0033$) than plants with one or no partners. While belowground biomass showed a similar pattern, it was not significant (Fig. S1B, Table S1, Supporting information; $F_{1,45}$=0.23, $P = 0.64$). The difference in above versus belowground effects may reflect different mutualist impacts on these two components of growth or may result from roots becoming pot-bound and reducing differences in growth. When we examined rhizobia nodulation, we found that nodules were absent in the R− treatments (0 ± 0 nodules), but common (32.13 ± 18.27 nodules) in the R+ plants ($F_{1,52} = 110.26$, $P < 0.0001$), with only two R+ plants failing to nodulate. Further, mycorrhizal fungi enhanced rhizobia nodulation by ~58% (Fig. 2C, Table S2, Supporting information; fungi × rhizobia: $F_{1,52} = 5.54$, $P = 0.0225$). There was also a trend towards heavier nodules in M+ roots, but it was not significant (Table S2, Supporting information; $F_{1,21} = 1.19$, $P = 0.2884$).

### *Transcriptome summary data and quality control*

For each of 8 replicate plants per treatment, we generated 24 319 187 ± 633 404 reads (total = 753 894 804 reads). One plant from the M+R− treatment was excluded before sequencing as the library did not pass quality control. Across all samples, ~83.63 ± 1.53% of reads mapped to the plant genome with more mapping when mycorrhizal fungi was absent (89.75 ± 0.40% of reads in M− treatments and 77 ± 2.07% of reads in M+ treatments; see Table S3, Supporting information for details on mapping rates for each sample). While 11.40% (±1.71) of reads mapped to the fungal genome for M + -treated plants, only 0.17% (±0.01) mapped to it for (M−)-treated plants, strongly suggesting that only plants grown in M+ treatments associated with the fungi (Fig. 2D, Tables S3, S4, Supporting information, fungi: $F_{1,27} = 403.19$, $P < 0.0001$). Further, we found that plants grown with both fungi and rhizobia had a greater percentage of reads mapping to the fungal genome (14.02 ± 1.70%) than in the treatment with just mycorrhizal fungi (8.41 ± 2.81%) suggesting that rhizobia may impact the abundance and/or expression of mycorrhizal fungi (Fig. 2D, Table S4, Supporting information; rhizobia: $F_{1,27} = 5.01$, $P = 0.0337$, fungi × rhizobia interaction: $F_{1,27} = 4.22$, $P = 0.0497$).

### *Do multiple mutualists have pervasive genomewide effects on expression?*

Clustering by treatment in the principal components analysis of the *M. truncatula* genomewide expression profiles indicated that both mycorrhizal fungi and rhizobia have substantial, large-scale effects on expression on many genes across the plant genome (Fig. 3). Further, we found a significant correlation between shoot biomass and the first two principal components axes, which explain >60% of the variation in expression (overall model: $R^2 = 0.27$, $F_{2,28} = 5.24$, $P = 0.0116$; PC1: $t_{1,28} = 2.44$, $P = 0.0212$; PC2: $t_{1,28} = 0.0422$). Differential expression analysis provided further insight into how

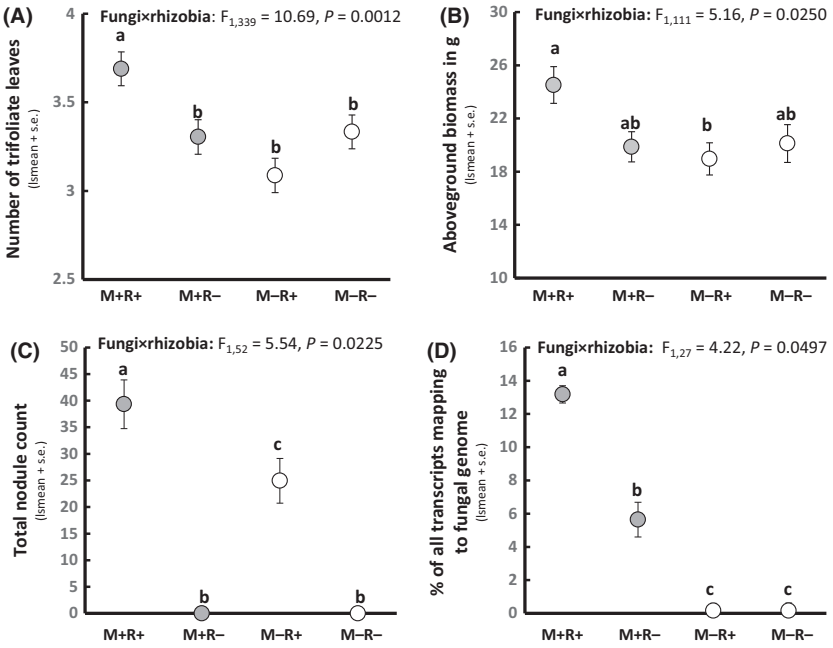


**Fig. 2** Effect of microbial treatments on plant performance, nodulation and per cent of transcriptional reads that map to the fungal genome. (A) Multiple mutualists had a synergistic effect on *M. truncatula* performance with M+R+ plants having significantly more trifoliate leaves throughout the experiment compared to other treatments (fungi × rhizobia: $F_{1,339}$ = 10.69, $P$ = 0.0012). (B) We observed similar results for aboveground biomass (fungi × rhizobia: $F_{1,111}$ = 5.16, $P$ = 0.0250) with M+R+ having significantly more mass than M+R- and marginally more mass than M+R− ($P$ = 0.0563) and M−R− ($P$ = 0.0739). (C) Nodules were absent in the R- treatments but common for R+ plants (rhizobia: $F_{1,52}$ = 110.26, $P$ < 0.0001) and that the presence of mycorrhizal fungi enhanced nodulation (fungi: $F_{1,52}$ = 5.54, $P$ = 0.0225, fungi × rhizobia: $F_{1,52}$ = 5.54, $P$ = 0.0225). (D) An average of 11% of all transcriptional reads mapped to the fungal genome for plants in the M+ treatments while an average of 0.17% of reads mapped to the fungal genome for M− treatment plants, suggesting that only plants in M+ treatments associated with fungi (fungi: $F_{1,27}$ = 403.19, $P$ < 0.0001). Further, the percentage of reads that mapped to the fungal genome was almost twice as much in the M+R+ as the M+R− (rhizobia: $F_{1,27}$ = 5.01, $P$ = 0.0337; fungi × rhizobia: $F_{1,27}$ = 4.22, $P$ = 0.0497). *All graphs display least-squares means ± standard error. Least-squares means in panel D are back-transformed after analysis of data with natural log-transformation (mean and standard error of raw data can be viewed within results section text).*

mutualists impacted expression of genes individually and together. We found a significant main effect of mycorrhizal fungi and rhizobia on expression of 4730 and 2627 *M. truncatula* genes, respectively (*i.e.* statistically significant main effects of the presence/absence of each partner), with 599 of these genes influenced by both partners (*i.e.* significant main effects of both rhizobia and fungi). We also detected 62 genes for which there was a significant interaction of the fungi and rhizobia, including 38 of the 599 genes with significant main effects for both partners. Even after correction for multiple comparisons, many of these significant genes had relatively small log2-fold changes (*i.e.* magnitude of the change in expression associated with microbial treatments) of <2 (see Table S5, Supporting information). Our experimental design's comparatively high degree of replication of samples within treatments and sequencing depth within samples gave us the power to detect these statistically significant, but quantitatively small, effects.

### *Do microbial mutualists predominantly impact gene expression individually, additively or nonadditively?*

*Individual Effects (expression affected by single mutualist)*: The majority of genes for which mutualism significantly impacted expression were affected by one partner or the other (*i.e.* ~4130 genes for mycorrhizal fungi and ~2030 genes for rhizobia; Table 1), meaning that these microbial mutualists not only impacted expression of most genes independently but also uniquely. The genes with expression affected by only the presence of fungi were significantly enriched for 18 GO terms (*i.e.* biological functions or processes) while those with expression affected only by rhizobia were enriched for 33 GO functions. Of these, one GO term was shared—catalytic activity (GO:0003824; rhizobia FDR = 0.035 and fungi FDR = 0.000000002). However, mycorrhizal presence was associated with hydrolase catalytic activity (GO:0016787; FDR = 0.0015), while rhizobial presence was associated with transferase catalytic activity

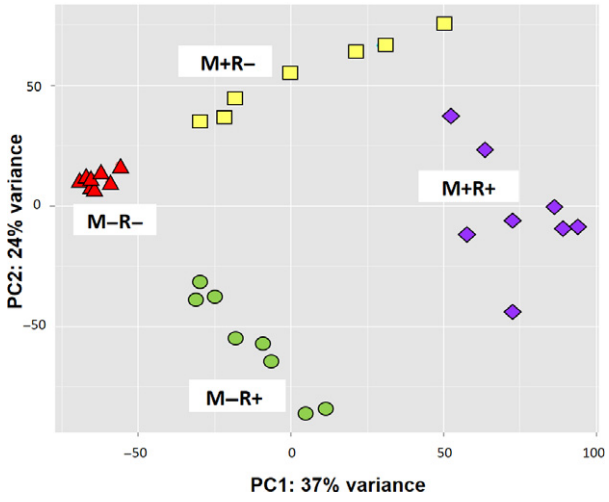


**Fig. 3** Principal components analysis of the *M. truncatula* genomewide expression profiles. The strong pattern of clustering by microbial treatment combination indicates that both mycorrhizal fungi and rhizobia have effects on expression across the genome. Each point indicates the transcriptome profile of an individual plant, and differences in the shape (or colour) of points indicate different treatment combinations. Each treatment combination has eight replicates (except M+R− which has seven replicates).

(FDR = 0.00078, GO:0016740). In particular, genes affected by mycorrhizal fungi were involved in several hydrolysis functions: hydrolysis of O-glycosyl bond (FDR = 0.0068) and serine-type carboxypeptidase activity (FDR = 0.014). The latter involves the hydrolysis of a peptide bond near the C-terminus of a polypeptide chain using a catalytic triad consisting of a serine nucleophile that is activated by a proton relay involving an acidic and a basic residue; serine carboxypeptidases can influence mycorrhizal development in the root cortex of *M. truncatula* (Rech *et al.* 2013). Further, mycorrhizal fungi impacted expression of genes involved in lipid and carbohydrate metabolic processing (*e.g.* FDR = 0.0045 and FDR = 0.0036). Rhizobia affected expression of genes with a diverse array of functions, including oxygen transport (FDR = 0.0000022), protein amino acid phosphorylation (FDR = 0.000005), ATP binding (FDR = 0.00064), secondary active transmembrane transporter activity (FDR = 0.035), protein serine/threonine kinase activity (FDR = 0.0000052) and protein tyrosine kinase activity (FDR = 0.00000059). More details on differentially expressed gene identities and enrichment results are available in Tables S5–S7 (Supporting information).

*Joint Effects (expression affected by both partners):* Although the expression of most genes was impacted by only one microbial mutualist, we found that there were still a substantial number of genes whose expression was impacted by both partners (*i.e.* jointly). In total, multiple mutualists affected the expression of 623 genes, ~10% of all differentially expressed genes (623 genes = 599 with main effects + 62 with interactions—38 shared).

To determine the different ways in which multiple mutualists can alter expression patterns, we examined these genes in detail. First, we classified effects as additive or nonadditive (Table 1). Additive effects included genes for which changes in expression associated with hosting both mutualists compared to the control equalled the sum of the changes in expression associated with each mutualist separately. We detected these statistically when there was a significant main effect of mycorrhizal fungi and of rhizobia but no significant interaction of these treatments (Table 1). Conversely, nonadditive effects indicated that changes in expression associated with multiple partners could not be predicted based on the pairwise treatments (*i.e.* expression changes with both is not the sum of changes in expression separately). We detected genes with nonadditive expression as those with significant interaction terms (Table 1). We found that multiple mutualists had additive effect on the expression of 561 (Fig. 4A; Table 1, Table S5, S8, Supporting information full list and statistical details) and nonadditive effects on the expression of an additional 62 genes (Fig. 4B–D; Table 1, Table S5, S9, Supporting information for list and details).

**Additive genes**, which made up ~90% of multiple mutualist-affected genes, were significantly (FDR = 0.03) enriched for acid phosphatase activity, a function that is important in plant phosphorus uptake and in turn plant growth (Duff *et al.* 1994; Xiao *et al.* 2006; Ma *et al.* 2012) as these enzymes help make the preferred form of environmental phosphorus ($P_i$, orthophosphate anions) available to plants via hydrolysis of orthophosphoric monoesters under acidic conditions (Duff *et al.* 1994). While in most cases (476 of the 561 additive genes) we found agreement over the direction of expression by both partners (*i.e.* both partners caused upregulation or downregulation in hosts when grown each partner separately; Fig. 1A), we did detect the potential for substantial conflict between partner species over the expression profile for 85 of the additive genes in that one partner significantly increased expression while the other significantly decreased expression compared to the control (Fig 1b., Tables S5, S8, Supporting information).

*Nonadditive genes* were enriched for several biological processes, including proteolysis (FDR = 0.028) and transport (FDR = 0.028), and several molecular functions, including transporter activity (FDR = 0.024) and cysteine-type peptidase activity (FDR = 0.00017), as well as related parent terms (*i.e.* localization, establishment of localization, peptidase activity and acting on L-amino

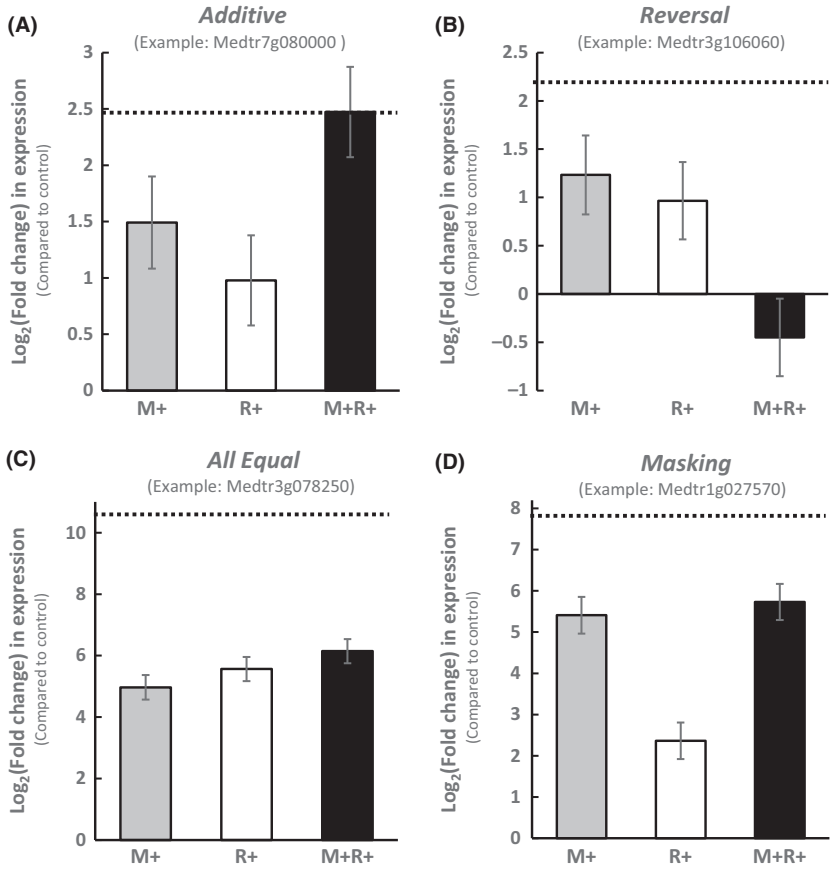


**Fig. 4** Observed categories of multiple mutualist effects on gene expression. (A) 'Additive' genes experience a change in expression in the presence of both mutualists that equals the sum of the changes in expression associated with each mutualist separately. (B) Genes experiencing 'reversals' are those for which expression changed direction in response to multiple mutualists, typically from upregulated when plants associated with a single partner to downregulated when plants interact with both partners. (C) 'All equal' genes experience a change in expression in the presence of multiple partners that is equal to the change in expression associate with the presence of either partner species alone (*i.e.* 95% confidence intervals around the M+, R+ and M+R+ means are all overlapping). (D) 'Masking' genes experience a change in expression with multiple partners equal to the change in expression in the presence of a single partner (*i.e.* 95% confidence intervals around the M+R+ mean overlaps 95% CIs around either the mean of M+ *or* R+) with the majority of these genes having changes in expression with multiple mutualists that equals changes in expression with just mycorrhizal fungi. Dashed line in all panels indicates additive expectation based on summing change in expression associated with single-mutualist treatments. Bars represent mean change in expression compared to control plus or minus standard error.

acid peptides). Full GO annotations, gene names and enrichment statistics are given in Table S10 (Supporting information). Within the set of nonadditive genes, we observed three main ways in which multiple mutualists impacted expression. While these categories are not completely mutually exclusive, most genes fell into a single category (*i.e.* only two nonadditive genes included in two categories). First, we found that ~30% of nonadditive genes (17 genes) were 'reversals': genes for which expression changed direction in response to multiple mutualists (Fig. 4B, Table S9, Supporting information). In nearly all cases of reversals, expression was upregulated when plants associated with a single partner but downregulated when plants interacted with both partners. These genes were significantly (FDR = 0.0118) enriched for *N*-acetyltransferase activity (as well as related parent terms) which is involved in transferring acetyl groups to nitrogen atoms on acceptor molecules (Binns *et al.* 2009). The opposite pattern of upregulation with multiple partners was only observed in one case, Medtr3g065250, which is a known glutamine synthetase gene (Li *et al.* 2012).

Second, ~20% of the nonadditive genes (12 genes) were 'all equal' in which changes in expression associated with multiple partners were equal to the change in expression associated with the presence of both partner species alone (Fig. 4C, Table S9, Supporting information). For these genes, expression levels were changed by the presence of mutualists but the effects were consistent regardless of identity (*i.e.* mycorrhizal fungi or

rhizobia) or number (*i.e.* one or multiple partners) of the mutualists. Within the 'all equal' genes, mutualistic associations led to upregulation in 3× as many cases as downregulation compared to the control treatment (Table S9, Supporting information). Enrichment analysis was not possible with this group because it contained a small number of genes and relatively few with annotation information, but we observed expression changes in genes that were particularly interesting (Table S9, Supporting information). For example, we observed upregulation of genes annotated as an auxin-induced 5NG4-like protein (Medtr2g102340) and a leucine-rich repeats (LRR) receptor-like kinase (Medtr3g078250). Previous work has identified another auxin-induced 5NG4-like protein (nodulin MtN21) implicated in the formation of nodules possibly through inducement of lateral root development (Gamas *et al*. 1996; Busov *et al*. 2004) and another LRR receptor-like kinase (SymRK/DMI2) as required for both mycorrhizal and rhizobial recognition (Stracke *et al*. 2002).

Third, 'masking' genes made up ~60% of the nonadditive genes (35 genes); 'masking' occurred when the change in expression with multiple partners equalled the change in expression with a single partner (Fig. 4D, Table S9, Supporting information). Nearly all of these genes had expression levels with multiple mutualists that were equal to expression in the presence of mycorrhizal fungi alone (~52% of the all nonadditive genes or 89% of all masking genes), and this set of genes was enriched for cysteine-type peptidase activity (FDR = 0.000609), protein serine/threonine kinase activity (FDR = 0.0229), protein amino acid phosphorylation (FDR = 0.035), protein metabolic processes (FDR = 0.0171), and transport of oligopeptides (FDR = 0.0171) and cations (FDR=0.035). We observed very few cases (three genes) of masking in which expression in the combined treatment equalled expression in the presence of rhizobia alone and these genes included annotations for formin-like 2 domain protein (Medtr3 g078623), disease resistance-like protein GS3-1 (Medtr6g072780) and IQ calmodulin-binding motif protein (Medtr7g114870).

### *Does one microbial partner have a stronger effect on gene expression than the other?*

Mycorrhizal fungi had a more important role in determining *M. truncatula* gene expression than rhizobia in our experiment. For ~61% of the 623 genes with expression significantly affected by multiple mutualists, changes in expression in the presence of both partners were more similar to the change in expression with mycorrhizal fungi alone than rhizobia alone ($\chi^2$=15.47, P = 0.00008; Fig. 5). Similarly, ~59% of all 36162 genes expressed during the experiment had changes in expression levels in the presence of both partners that were more similar to changes in expression levels with mycorrhizal fungi alone than rhizobia alone ($\chi^2$ =570.1, $P < 0.00001$). Finally, approximately 70% of the significant genes in the differential expression analysis were affected by mycorrhizal fungi, and 60% of the significant genes were only affected by mycorrhizal fungi while ~30% of significant genes were only affected by rhizobia.

### *Does multispecies mutualism affect gene expression in one of the microbial partners?*

We observed expression in ~70% of genes across the mycorrhizal genome (20 912 of 30 282 genes) with more genes being upregulated (11 701) than downregulated (9211) in the presence of rhizobia ($\chi^2$ = 148.77, $P < 0.00001$). However, we were only able to detect rhizobia-caused significant changes in expression in a few mycorrhizal genes, probably due to the relatively small number of reads mapping the mycorrhizal genome (~11% of reads or <100 reads/per gene for each root sample). In particular, we found that two genes with protein IDs 30454 (adj. $P = 0.0057$; gene ID: gm1.30454_g) and 22556 (adj. $P = 0.0100$; gm1.22556_g) were significantly upregulated when rhizobia was present. While the second gene lacks any annotation, the first is associated with GO terms for inorganic anion transmembrane transporter activity and related terms and has been characterized in mycorrhizal fungi as a

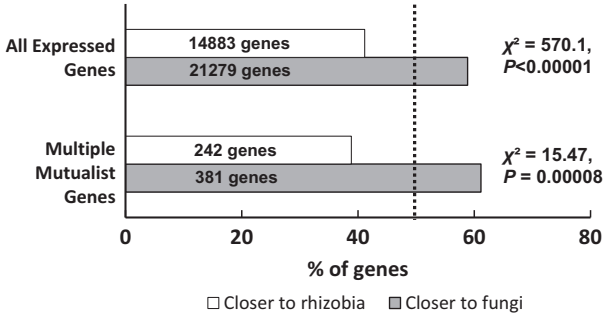


**Fig. 5** Mycorrhizal fungi have a greater effect on *M. truncatula* gene expression than rhizobia. Approximately 59% of all 36 162 expressed genes showed changes in expression with both partner that were more similar to changes in expression with mycorrhizal fungi alone than rhizobia alone ($\chi^2$ = 570.1, $P < 0.00001$). For the set of 623 genes with expression affected by multiple mutualists, similar results were found (61% genes closer to mycorrhizal levels of expression; $\chi^2$ = 15.47, $P = 0.00008$). Dotted line marks the 50% expectation: 50% of genes expected to have changes in expression with multiple partners that is closer to expression with rhizobia and 50% expected to have changes in expression closer to that with mycorrhizal fungi.

nitrate transporter involved in primary N assimilation that precedes N transfer to plants (Tian *et al.* 2010).

## Discussion

Studies of mutualisms have demonstrated their importance for myriad ecological and evolutionary processes (Fontaine *et al.* 2006; Rudgers *et al.* 2007; Joy 2013; Keller 2014b; Weber & Agrawal 2014), but have often failed to incorporate multispecies interactions, which are extremely common in nature and may dramatically alter the outcomes for the participants and higher-level processes (Afkhami *et al.* 2014; van der Heijden *et al.* 2016). Unravelling the consequences of multispecies mutualisms requires both an understanding of their effect on the performance of participants and the molecular mechanisms underlying them, yet to our knowledge, the latter has not been previously examined with consideration of interactive, nonadditive effects. We combined manipulative experiments and genomewide expression analysis to determine phenotypic and transcriptomic responses of *Medicago truncatula* to its interaction with rhizobia and mycorrhizal fungi. Our work revealed four key findings: (i) rhizobia and mycorrhizal fungi synergistically improved plant performance and caused pervasive genomewide effects on expression. (ii) Expression was most often influenced by a single mutualist; however, ~10% of differentially expressed genes were affected by both partners including unexpected nonadditive outcomes like reversals in the direction of expression with two—rather than one—symbionts. (iii) Fungi had a significantly stronger effect on genomewide plant expression than rhizobia. (iv) The presence of one partner (rhizobia) altered expression of another partner (mycorrhizal fungi).

### *Performance effects of multiple mutualists*

Our finding of synergistic effects of rhizobia and mycorrhizal fungi on plant performance is consistent with numerous past suggestions and has potentially broad ecological implications, although relatively few studies have conducted factorial manipulations of both partners to test for interactive effects (Larimer *et al.* 2010). For years, researchers have recognized the importance of microbial mutualists, like rhizobia and arbuscular mycorrhizal fungi, for plant success and in some cases even the potential import of interactions among these symbionts for vital ecological and evolutionary processes (Herrera 1993; van der Heijden *et al.* 2016). The biology of rhizobia and mycorrhizal interactions suggest a wide range of outcomes may occur; complementarity of the primary rewards conferred by these partners (*i.e.* nitrogen fixation and phosphorus/water acquisition) could lead to synergistic effects on performance while the similarity of the primary reward received (*i.e.* photosynthetic carbon) could lead to conflict with cascading negative effects for hosts (Afkhami *et al.* 2014). A meta-analysis found that growing plants with both N-fixing bacteria and mycorrhizal fungi typically has positive, additive effects on plant performance (Larimer *et al.* 2010), but more recent studies documented synergistic consequences of these mutualists in other legume species (Larimer *et al.* 2014; van der Heijden *et al.* 2016). Recent work has also revealed that complementarity among rhizobia and mycorrhizal fungi can have cascading effects for key community and ecosystem-level processes. For example, in microcosms, interactions with both symbionts resulted in increased plant diversity, recruitment and nutrient acquisition as well as shifts in community composition (van der Heijden *et al.* 2016). Given their role in N and P cycles, the complex interactions we documented among rhizobia and mycorrhizae could have cascading effects on ecosystem processes (van der Heijden *et al.* 2008).

While pairwise interactions between the model legume *Medicago truncatula* and each of these microbial mutualists have been studied intensively, to our knowledge, no previous studies have manipulated both of these interactions simultaneously in this system. Our results, revealing a synergistic effect of rhizobia and mycorrhizal fungi on *M. truncatula* grown in nutrient-poor conditions, provide an excellent opportunity for investigating the molecular basis of synergistic interactions. In the long term, this type of microbial synergism research may be useful for applied goals like restoring natural ecosystems or even agricultural plant improvement, especially given *M. truncatula* is a close relative of alfalfa (*Medicago sativa*) that also associates with both rhizobia and mycorrhizal fungi (Van Rhijn *et al.* 1997). For example, we found several lines of evidence from transcriptomic data suggesting that the presence of both partners had important consequences for nutritional provisioning of the host plants. The presence of rhizobia led to upregulation of a nitrate transporter in mycorrhizal fungi that is involved in primary N assimilation by the fungus which precedes N transfer to plants (Tian *et al.* 2010). Further, plant functions involved in nutrition were also influenced by both partners. For example, acid phosphatase activity, which is important for phosphorus uptake and in turn plant growth (Duff *et al.* 1994; Xiao *et al.* 2006; Ma *et al.* 2012), was enriched among the genes affected by both microbes. These mutualists also altered expression of important enzymes involved in nutrient acquisition. For example, 30% of genes with clear annotations for glutamine synthetase in *M. truncatula* from a search of the database LegumeIP (Li *et al.*

2012) were affected by these symbionts, and one gene (Medtr3g065250) was affected nonadditively with expression being upregulated synergistically in the presence of multiple partners. Because glutamine synthetase is the first enzyme in the main pathway of ammonium assimilation in higher plants, it a key target for improving nitrogen use efficiency in crop species (Miflin & Habash 2002; Seabra & Carvalho 2015) and illustrates the potential importance of understanding interactive effects of partner species on the molecular phenotype. While beyond the scope of our global analyses of expression patterns, follow-up work with methods like RT–PCR could be important for validation of promising genes like these and is a critical next step in understanding the link between phenotypic and molecular consequences of multiple mutualists and applied goals.

### *Pervasive genomewide effects on expression*

We found extensive and pervasive effects of microbial mutualists on expression of many genes across the genome (i.e. genomewide), so much so that we could easily differentiate plant treatments based on the expression profiles in our multivariate analyses. Clear distinctions among all four treatments in expression (Fig. 3) were in contrast to traditional phenotypic measurements, in which the M+R+ treatment was often the only one significantly different from the others (Fig. 2). This contrast between molecular and traditional phenotypes may stem from a lack of concordance between gene expression and proteins, resulting in buffering of traits from molecular perturbations (Fu *et al.* 2009). At minimum, our data demonstrate that substantial plasticity in the plant transcriptome is occurring in response to changes in the biotic community, including 'hidden players'. It is also likely that these shifts in expression altered plant performance and size-related traits in some cases. The significant correlation we detected between genomewide expression and shoot biomass supports a link between microbial-mediated molecular and performance phenotypes, but future work examining expression changes across the genome in leaves and shoots could be invaluable for unravelling this relationship. While our experimental design had the power to detect quantitatively small expression differences (judged by log-fold change), other evidence suggests that these changes in aggregate had biologically significant consequences. For example, the substantial divergence of plant expression profiles by microbial treatment in the multivariate analysis and the significant relationship between genomewide expression and biomass suggests that the changes in expression that we observed across a large number of genes may have important cumulative effects on the molecular and performance phenotypes.

Two additional aspects of the global gene expression patterns stand out to us. First, perhaps not surprisingly, diverse transcriptomic and molecular phenotypes can underlie a single whole-plant phenotype. For instance, leaf number, biomass and branch number showed little effect of rhizobia or fungi alone, but clear effects of both together. We saw few gene expression profiles matching these phenotypic trends, illustrating how diverse mechanistic bases can underlie a single pattern in 'whole-plant' phenotypes like leaf or branch number. Interestingly, the genes whose expression was upregulated illustrate potential mechanisms by which a synergistic, nonadditive phenotypic effect can be achieved with additive gene expression. For example, both rhizobia and fungi had additive effects on phosphatase activity, an important function in P uptake and metabolism. Enhanced P uptake, perhaps from increased expression of these genes, combined with fixed N from nodules, could lead to synergistic, nonadditive effects on plant growth and performance, even in the absence of individual genes showing a synergistic, nonadditive pattern of expression. Selection on whole-plant performance traits, in turn, might have very difficult to predict consequences on the evolutionary dynamics of these genes, given the diverse expression profiles that underlie them.

Second, we hypothesized that expression in some genetic pathways might be subject to conflict: if one mutualist leads to increased expression, while another leads to decreased expression, the net expression when both are present may be subject to conflict (Fig. 1b). While in most cases—~85% of additive genes—we observed agreement among partners over the direction of regulation (*i.e.* Fig. 1a), we did observe expression profiles for 85 additive genes that could indicate conflict in response to multiple mutualists. Previous phenotypic studies also suggest a complex relationship between mycorrhizal fungi and rhizobia: nodule number can be enhanced by the fungi (Lekberg & Koide 2005; Wang *et al.* 2011; Ossler *et al.* 2015; Fig. 2C), but under some conditions, trade-offs between fungal colonization and nodule number may occur (Catford *et al.* 2003; Sakamoto *et al.* 2013). Within this context, nonadditively expressed genes showing patterns of masking or reversals may also be indicative of conflict or complementarity between mutualists. For example, almost all of our masking genes had expression in co-inoculation more similar to the mycorrhizal fungi treatment than to the rhizobial treatment, suggesting that fungal infection had a greater influence on gene expression. Reversals, in which expression is increased in response to one mutualist, but decreased in response to two mutualists may

reflect nutritional or reward complementarity between mutualists obviating the need for expression on part of the plant. At minimum, these reversals indicate that the simple chain of logic connecting expression levels to phenotypic trends (i.e. as if phenotypes were a simple, linear function of expression) clearly breaks down in response to multiple interactors.

### *A strong role for mycorrhizae*

While both bacterial and fungal partner species had pervasive consequences for expression of their host, our data suggested a stronger impact of mycorrhizal fungi on plant transcriptomes. In fact, expression of almost twice as many genes was affected by mycorrhizal fungi compared with rhizobia (4760 and 2657 genes, respectively), which is especially interesting in the light of how much focus has been given to legume–rhizobia symbioses (Hirsch *et al.* 2001). However, it is important to keep in mind that transcript number dominance may or may not equal phenotypic or ecological dominance: changes in expression in a few genes caused by rhizobia might be more important for fitness and a plant's ecological dynamics than changes in expression across many genes caused by mycorrhizal fungi.

The predominance of mycorrhizal fungi's effects on expression may be due to several nonexclusive explanations. For example, differences in spatial or temporal colonization patterns between symbionts could be important. Specifically, separation of rhizobia in nodules may allow for more localization of changes in expression compared to mycorrhizal fungi, which may be root-wide. Mycorrhizal fungi might also have been slower to colonize, but with longer-lasting effects, while strong rhizobial effects could be temporally restricted to root colonization or nodule formation periods. It is also possible that the longer evolutionary history of symbiosis between mycorrhizal fungi and plants (Humphreys *et al.* 2010) compared to that between rhizobia and plants could influence the strength of its effects on expression. Finally, ubiquitous mycorrhizal effects on expression could have resulted from fungi conferring multiple resources to plants. While fitness benefits of mycorrhizal fungi are often tied to phosphorus provisioning, they can also provide other benefits like increased nitrogen and water uptake (Khalvati *et al.* 2006; Bücking & Kafle 2015). Unlike rhizobia that access a unique atmospheric nitrogen pool, mycorrhizal fungi accumulate soil nitrogen, and they are thought to make a relatively greater contribution to plant phosphorus uptake than nitrogen uptake (George *et al.* 1995). In the nutrient-poor conditions of this experiment, the pool of nitrogen available to rhizobia (atmospheric N) was much greater than that available to mycorrhizal fungi (soil N), and water was highly available to all plants regardless of treatment. However, even minor effects of fungi on these resources may have significant impacts on the plant's molecular phenotype (Govindarajulu *et al.* 2005). Distinguishing between these possibilities for the predominance of mycorrhizal effects on expression would require characterizing gene expression on a finer spatial scale in the organisms (*i.e.* near and far from nodules in roots), at multiple temporal points (immediately after inoculation versus later in the life cycle), under different resource environments (*e.g.* high versus low N and P) and possibly by comparing expression profiles in multispecies interactions that differ in their length of shared evolutionary history.

Interestingly, we see similar patterns for symbiont signalling through the 'common symbiosis pathway' that is involved in both nodulation and the formation of mycorrhiza. The signalling pathway as described for *M. truncatula* in Oldroyd (2013) has at least 12 genes for which we have expression data. They include five genes believed to be specific to nodulation (LYK3, NFP, NSP1, NIN and ERN1), two specific to mycorrhiza formation (RAM1 and RAM2), and five used in the formation of both associations (DMI1, DMI2, DMI3, IPD3 and NSP2) (Markmann & Parniske 2009; Oldroyd *et al.* 2009; Oldroyd 2013). In our data, only mycorrhizal fungi significantly impacted expression of any of the genes previously identified as involved in the formation of both associations (three of five genes; DMI1: $P = 0.009$, DMI2: $P = 0.054$, NSP2: $P < 0.0001$; Table S11, Supporting information). In fact, mycorrhizal fungi also not only caused significant upregulation of those shared genes and the two genes specific to mycorrhizal colonization (RAM1: $P \ll 0.0001$; RAM2: $P \ll 0.0001$; Table S11, Supporting information), it even significantly influenced expression of two of the five genes that are thought to be part of nodulation-specific signalling. The fungal symbiont caused downregulation of NFP ($P = 0.015$), which is involved in plant recognition of nod factor (a signal produced by the bacteria), and upregulation of NSP1 ($P \ll 0.0001$), which is a GRAS domain transcription factor that functions downstream of the shared genes and is involved in the formation of nodules (Oldroyd 2013). Conversely, rhizobia only significantly impacted the expression of genes specific to nodule formation (four of five nodule genes; see Table S11, Supporting information). These findings further support the hypothesis that the formation of mycorrhizal symbioses may have widespread and long-lasting effects on transcription of the symbiosis pathway. In examining the subset of genes involved in signalling, we also noted that (i) there were no significant interactive effects on the expression of the common symbiosis pathway, (ii) we rarely observed significant

additive effects (1 of 12 genes), and (iii) at least one partner influenced expression of most genes (10 of 12 genes). These findings highlight several important questions about how often signalling through the common symbiosis pathway is used simultaneously by both rhizobia and fungi, and whether interactive effects on the symbiosis pathway are present at other stages of development. Both of these possibilities could be investigated in future work on expression of the pathway across ontogeny.

### *Conclusions and prospects*

Our experiment clearly shows multiple mutualist effects in the rhizobia-mycorrhizal fungi–legume system, and our results from transcriptomic analyses support the hypothesis that nutritional complementarity is important in MMEs. This study also demonstrates that plants face clear cases of potential conflict when interacting with multiple mutualists and that perhaps mycorrhizal fungi play a more important molecular role in this tripartite mutualism than previously appreciated. Although multifactorial studies of gene expression in response to different biotic interactors are few, we predict that the pervasive and diverse transcriptomic responses underlying whole-plant responses that we detected are likely to be common, with important cascading effects for a broad range of ecological and evolutionary processes.

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MEA and JRS designed the experiment. MEA conducted the experiment, analysed the data and drafted the manuscript with substantial contributions from JRS on all aspects.

## Data accessibility

Expression data are available at NCBI's Sequence Read Archive (SRP078249) and phenotypic data available through Dryad (doi:10.5061/dryad.8f66s).

## Supporting information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Effect of microbial mutualists on stem branching (A) and belowground biomass (B).

**Table S1** Statistical results from plant growth data collected during the experiment and at harvest.

**Table S2** Statistical results from rhizobia nodulation data.

**Table S3** Reads mapping the plant and fungal genomes

**Table S4** Statistical results from ANOVA of percent of transcriptional reads mapping to fungal genome.

**Table S5** Change in plant expression associated with microbial treatments.

**Table S6** Genes with expression affected by single mutualist.

**Table S7** Enrichment analysis for genes with expression affected by rhizobia OR mycorrhizal fungi.

**Table S8** Genes with expression affected additively by multiple mutualists (*i.e.,* significant main effects of each mutualist but no significant interaction).

**Table S9** Genes with expression affected non-additively by multiple mutualists (*i.e.,* significant interaction of rhizobia and fungi).

**Table S10** Enrichment analysis for genes affected by multiple mutualists.

**Table S11** "Common Symbiosis Pathway" genes.