



# Cooperation and coexpression: How coexpression networks shift in response to multiple mutualists

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**Abstract**

A mechanistic understanding of community ecology requires tackling the nonadditive effects of multispecies interactions, a challenge that necessitates integration of ecological and molecular complexity—namely moving beyond pairwise ecological interaction studies and the “gene at a time” approach to mechanism. Here, we investigate the consequences of multispecies mutualisms for the structure and function of genomewide differential coexpression networks for the first time, using the tractable and ecologically important interaction between legume *Medicago truncatula*, rhizobia and mycorrhizal fungi. First, we found that genes whose expression is affected nonadditively by multiple mutualists are more highly connected in gene networks than expected by chance and had 94% greater network centrality than genes showing additive effects, suggesting that nonadditive genes may be key players in the widespread transcriptomic responses to multispecies symbioses. Second, multispecies mutualisms substantially changed coexpression network structure of 18 modules of host plant genes and 22 modules of the fungal symbionts’ genes, indicating that third-party mutualists can cause significant rewiring of plant and fungal molecular networks. Third, we found that 60% of the coexpressed gene sets that explained variation in plant performance had coexpression structures that were altered by interactive effects of rhizobia and fungi. Finally, an “across-symbiosis” approach identified sets of plant and mycorrhizal genes whose coexpression structure was unique to the multiple mutualist context and suggested coupled responses across the plant–mycorrhizal interaction to rhizobial mutualists. Taken together, these results show multispecies mutualisms have substantial effects on the molecular interactions in host plants, microbes and across symbiotic boundaries.

**KEYWORDS**

mycorrhizal fungi, nonadditive, rhizobia, RNA-Seq, symbiosis, transcriptome, weighted gene coexpression network analysis

## 1 | INTRODUCTION

As members of complex communities, organisms face diverse interactions throughout their lifecycle that strongly shape their traits, life history strategies, behaviours and fitness, often in complicated, non-additive ways (Afkhami, Rudgers, & Stachowicz, 2014; Herrera,

2000; Lively, de Roode, Duffy, Graham, & Koskella, 2014; McKeon, Stier, McIlroy, & Bolker, 2012; Pohl, Carvalho, Botto-Mahan, & Medel, 2006; Sih, Englund, & Wooster, 1998; TerHorst et al., 2015). Organisms are enmeshed not only in a matrix of negative associations, like competition and predation, but also positive interactions with diverse and ubiquitous mutualistic communities that provide

crucial benefits. A growing number of empirical studies have shown that the effects of multiple mutualists on host fitness and traits can be nonadditive (Afkhami & Stinchcombe, 2016; Brittain, Williams, Kremen, & Klein, 2013; Gustafson & Casper, 2006; Lau & Galloway, 2004; Ossler, Zielinski, & Heath, 2015; Palmer et al., 2010). For example, Stachowicz and Whitlatch (2005) detected synergistic effects of two gastropod species on the growth of their algal host; because each gastropod partner defended the alga against a distinct group of herbivores, both gastropods were required for algal growth. Theoretical and empirical work suggests that the nonadditivity generated by multiple mutualistic interactions can result from complementary rewards from distinct partners that lead to synergistic effects on the host's growth. However, nonadditivity due to multiple mutualistic interactions can also lead to very costly, antagonistic effects. In the latter case, competition among partner species over access to a limited host-provided resource could result in reduced partner efficacy or even host exploitation (Afkhami et al., 2014; Stanton, 2003).

Understanding how organisms regulate interactions in communities in which nonadditive outcomes are common is a considerable challenge that requires study and integration across many levels of biological organization. In community ecology, theory and empirical studies strive to determine the environmental factors (Warfe & Barmuta, 2004), natural history characteristics of participating species (Palmer et al., 2010) and other ecological mechanisms (Griffen & Byers, 2006) that lead to different types of nonadditive and additive outcomes of multispecies interactions. For instance, species and genotype diversity of plant communities can generate substantially higher productivity, resilience and stability of communities and ecosystems due to resource partitioning and facilitation among taxa (Cardinale, Palmer, & Collins, 2002; Crawford & Rudgers, 2012; Crutsinger et al., 2006; Hughes, Inouye, Johnson, Underwood, & Vellend, 2008; Tittensor et al., 2014). Similarly, interaction network and demographic studies suggest that generalist species that associate with multiple mutualistic partners have higher fitness than specialists when there is spatial or temporal heterogeneity in partner availability/quality and that a few generalist species can be critical for the broader community's stability (Afkhami et al., 2014; Bascompte, 2009; Palmer et al., 2010).

Our understanding of the ecological mechanisms and consequences of multispecies interactions greatly exceeds our understanding of the physiological, genetic and molecular mechanisms underlying how any individual species interacts with multiple partners. There are two major stumbling blocks to making progress on this goal. First, addressing this question requires studying the molecular response and genetic basis of interactions with multiple partners. The growing molecular and genomic resources for plants and microbes, combined with the accompanying ecological literature (e.g., Larimer, Bever, & Clay, 2010; Larimer, Clay, & Bever, 2014; Ossler et al., 2015; van der Heijden, Bardgett, & van Straalen, 2008), provide an opportunity to tackle this first challenge. Indeed, some studies of tripartite microbial symbioses have begun by identifying overlap in genes that are induced or suppressed in pairwise

interaction studies (Deguchi et al., 2007; Manthey et al., 2004; Salzer et al., 2000; Tomas et al., 2012). For example, two plant nutritional mutualists—N-fixing bacteria and mycorrhizal fungi—both induced genes involved in functions like membrane transport, primary metabolism and cell wall construction in separate experiments (Manthey et al., 2004) and use a shared “common symbiosis pathway” for establishment of their interaction with the plant (Markmann & Parniske, 2009; Oldroyd, 2013). Second, many of the existing genetic and transcriptomic studies of multispecies mutualisms (including our own: Afkhami & Stinchcombe, 2016) have necessarily taken a “gene at a time” approach. Considering each gene in isolation (e.g., its expression) fails to consider how multispecies interactions will affect the expression and function of multiple genes simultaneously. A network approach has the potential to add a complementary perspective, in that it can identify suites of genes whose expression change in concert in response to single or multiple mutualists.

A network perspective on the transcriptomic response to multispecies mutualisms is likely to be complementary to a “gene at a time” approach in several important ways. Identifying suites of genes with correlated expression will make it more tractable to identify groups of genes whose collective expression is associated with complex, ecologically relevant outcomes (see Des Marais, Guerrero, Lasky, & Scarpino, 2017 for an example related to plant drought and cold responses). These genes can then be studied in their own right—for example, their molecular population genetic history (Josephs, Wright, Stinchcombe, & Schoen, 2017), for agricultural purposes, or as tools for further probing the structure of molecular pathways. Network approaches also provide greater context of potential gene–gene interactions for those genes whose expression is differentially affected additively or nonadditively by multiple mutualists. Are these genes at the periphery of gene expression networks, with their expression responsive to single or multiple mutualists because they are relatively unconnected to other genes? Or, alternatively, are these genes highly connected to many other genes, making them potentially key players in widespread transcriptomic responses to complex mutualisms?

Here, we investigate the consequences of multispecies mutualisms for the structure and function of genomewide expression networks for the first time, using the tractable and ecologically important interaction between plants, rhizobia and mycorrhizal fungi. First, we examine a set of genes previously determined to show additive and nonadditive transcriptomic responses to multiple mutualists, asking: (i) Are genes nonadditively affected by multiple mutualists more highly connected? We then examine how the context of multispecies mutualisms affects coexpression networks of plant genes, microbial mutualist gene expression and gene expression across the plant–microbial symbiosis to develop a more complete picture of how a multispecies community context affects organisms' molecular phenotype. Specifically, we ask: (ii) Do multispecies mutualisms substantially change gene coexpression networks of hosts? (iii) Do sets of host genes that are differentially coexpressed in response to microbial mutualists explain variation in plant performance? (iv) How is fungal gene coexpression affected by rhizobia? (v) How is

the joint coexpression of plant and fungal genes affected by the presence of rhizobia?

## 2 | MATERIALS AND METHODS

### 2.1 | Study system

We used *Medicago truncatula* (Jemalong A17) and two microbial symbionts—mycorrhizal fungi (*Rhizophagus irregularis* DAOM197198) and rhizobia (*Ensifer meliloti* Rm1021)—to study the effects of tripartite interactions between a host and its symbionts on genomewide coexpression networks. *Medicago truncatula* is a common model system for studying symbioses because (i) it has substantial genomic resources (Branca et al., 2011; Burghardt et al., 2017; Friesen et al., 2014; Tang et al., 2014; Yoder et al., 2014) and (ii) interacts with two of the most common microbial mutualists—mycorrhizal fungi and rhizobia (Mathesius, Journet, & Sumner, 2006). While natural frequencies of interactions between *M. truncatula* and each of its microbial partners in nature are needed, experimental manipulations such as ours (that simulate the extremes of variable bacterial and fungal densities) in *M. truncatula* make an excellent system for understanding the molecular basis of multiple mutualist effects. Previous work in this system has documented genes that are differentially expressed in response to each of these mutualists individually as well as together (Afkhami & Stinchcombe, 2016). That differential expression study found that while expression of most genes was affected by one symbiont or the other, the expression of 623 genes was significantly altered by multiple mutualists. In most cases (561 genes), the multiple mutualists had an additive joint effect on the expression such that the effects of the two mutualists on expression are independent and the effects of multiple mutualists can be calculated by summing effects with each mutualist. However, these mutualists had nonadditive effects on an additional 62 genes, where the effects of one mutualist on expression are influenced by the presence of the other, and the effects of multiple mutualists cannot be calculated by summing effects with each mutualist. The latter group included unexpected results, like 17 genes whose expression switched direction in the presence of multiple mutualists compared to a single partner.

### 2.2 | Experimental design and data collection

Because experimental methods have been previously described in Afkhami and Stinchcombe (2016), we give only a brief overview here. One hundred and twenty *M. truncatula* JA17 were grown in a complete randomized block design experiment manipulating rhizobia and mycorrhizal fungi. Microbial environments were as follows: only rhizobia (M–R+), only mycorrhizal fungi (M+R–), both (M+R+) or neither (M–R–). We inoculated sterilized nested magenta boxes containing triple autoclaved sand (Heath, Stock, & Stinchcombe, 2010) at 1 and 1.5 weeks after germination. R+ plants were inoculated with  $\sim 10^7$  rhizobia cells in sterile ddH<sub>2</sub>O and R– plants with 1 ml sterile ddH<sub>2</sub>O (Simonsen & Stinchcombe, 2014). Similarly, we treated

M+ plants with  $\sim 300$  spores in sterile ddH<sub>2</sub>O and M– plants with the same inoculum after autoclaving ( $4\times$  at 121°C) to ensure spore inviability (Antunes, Miller, Carvalho, Klironomos, & Newman, 2008; Powell et al., 2009). Plants were harvested at 7 weeks, after symbiosis establishment but prior to transcriptional changes associated with plant senescence. We rinsed (in sterile water), flash-froze, homogenized and then extracted RNA from whole root systems of 32 plants (8 per treatment; Norgen Biotek's Plant/Fungi Total RNA Purification Kit), and dried and weighed aboveground tissues of all plants. We characterized RNA with a Nanodrop 1000 ( $132.38 \pm 9.84$  ng/ $\mu$ l) and a 2100 Bioanalyzer (RNA Integrity Numbers =  $9.18 \pm 0.06$ ; max possible RIN = 10) and constructed cDNA libraries (TruSeq Stranded mRNA Sample Preparation Kit). One M+R– library was excluded as it did not pass quality control. Since rhizobial mRNA lacks poly-A tails, no rhizobia expression data were produced. We sequenced libraries on the Illumina HiSeq 2000 with 100-bp paired-end reads (8 samples/lane; Genome Quebec at McGill University). We verified that inoculations were successful by confirming the presence of nodules for the rhizobial treatment and of transcripts mapping to the fungal genome for the mycorrhizal treatment (Afkhami & Stinchcombe, 2016).

### 2.3 | Data processing

We used TOPHAT (version 2.0.12), allowing for two mismatches per read, with BOWTIE (version 2.2.3) (Langmead & Salzberg, 2012; Trapnell, Pachter, & Salzberg, 2009) to map and align the sequence reads to the *M. truncatula* JA17 and *R. irregularis* DOAM 197198 genomes. We then determined the number of reads per gene for each sample using HTSEQ (version 1.8.1; Anders, Pyl, & Huber, 2015), followed by filtering and removal of lane effects (LIMMA package; Smyth, 2005). Because samples were randomized across sequencing lanes with respect to microbial treatment, lane effects are not confounded with treatments. We included all genes for which read counts were  $>10$  in at least one of the samples within at least one treatment group (Sha, Phan, & Wang, 2015). Results were robust to different filtering cut-offs, producing qualitatively similar results (Figure S1). Following weighted gene coexpression network analysis (WGCNA) best practices (Langfelder & Horvath, 2017), read counts per gene were transformed to correct for RNA-seq bias towards genes with high expression levels with the `rlog()` function in DeSeq2 (Langfelder & Horvath, 2008; Love, Anders, & Huber, 2014) which produces a transformation to the log<sub>2</sub> scale while normalizing for the number of reads in each sample. We then quantile normalized with the `preprocessCore` package (Bolstad, 2013) following guidance of Hicks & Iriazary, 2014 (Figure S2).

### 2.4 | How connected are genes affected by multiple mutualists? Centrality of additively vs. nonadditively affected genes in expression network

We constructed an undirected weighted network with the nodes as genes and edges as Pearson's correlation values of expression levels.

We then calculated the degree centrality, the sum of all the edges connecting the node to the rest of the network:

$$C_D(i) = \sum_j^N r_{ij}$$

where  $i$  is the focal gene,  $j$  is every other gene,  $N$  is the total number of genes, and  $r$  is the absolute value of the Pearson's correlation between gene  $i$  and gene  $j$  raised to a power of 10 to fit a scale-free-network topology (Barabási, 2013). Biologically, degree centrality (centrality hereafter) gives a measure of how correlated each gene's expression is to the expression of other genes in the network; we interpret this as a metric of how connected a gene is to the rest of the network. Degree centrality has also been shown to be a metric of biological importance; for example, proteins with high-degree centrality are more essential for survival and have slower evolutionary rates than those with low centrality (Hahn & Kern, 2005; Koschützki & Schreiber, 2008). We calculated and compared the degree centrality of genes previously identified (Afkhami & Stinchcombe, 2016) to show additive and nonadditive expression in the response to multispecies mutualisms. For comparison, we generated the degree centrality of randomly sampled genes from the network and calculated the  $z$ -value of the degree centrality of additive and nonadditive genes ( $n = 1,000$ ). To determine whether nonadditive and additive genes had significantly different centralities, we randomly sampled 62 additive genes (without replacement) to match the sample size of nonadditive genes and used a  $t$  test to compare the mean centrality of the additive and nonadditive gene sets. We repeated this 1,000 times, saving the  $t$ -value from each test, and then examined what proportion of tests yielded a significant difference in centrality. We interpreted differences in centrality as non-significant if more than 5% of these  $t$  tests with equalized sample sizes were not significant.

## 2.5 | Detecting and characterizing differentially coexpressed genes

To identify how microbes change coexpression, we used differential coexpression analysis which identifies clusters or "modules" of genes whose coexpression network changes among treatments (here microbial environments). Our goal was to identify suites of genes whose expression correlations differed between treatments which could provide insights into the functional basis of multispecies mutualisms and the molecular mechanisms that underlie them. With the transformed, normalized genes (of the plant, mycorrhizal fungi, or both) as the input, we utilized WGCNA (Langfelder & Horvath, 2008) with the DiffCoEx add-on (Tesson, Breitling, & Jansen, 2010) to determine the consequences of mutualist and multiple mutualist effects for coexpression of genes in the shared plant host, in the mycorrhizal fungi, and across the symbiosis (i.e., plant and fungi genomes).

The DiffCoEx computational pipeline generates modules using all samples, based on a distance matrix reflecting biological differences

caused by our experimental treatments. To do this, it first identifies coexpression relationships among genes by estimating a Spearman's rank correlation matrix between all pairs of genes within each treatment (Tesson et al., 2010). To call differentially coexpressed modules, the algorithm compares how coexpression relationships change across all four treatments by generating a distance matrix, where each entry was the difference in rank correlations of expression across treatments (Tesson et al., 2010). We used the generalized form of the distance metric outlined below which allows us to account for the four treatments when determining differentially coexpressed modules:

$$D : d_{ij} = \left( \sqrt{\frac{1}{n-1} \sum_k \frac{|\text{sgn}(c_{ij}^{[k]}) * (c_{ij}^{[k]})^2 - c_{ij}^{[0]}|}{2}} \right)^\beta$$

with

$$c_{ij}^{[0]} = \frac{1}{n} \sum_k \left( \text{sgn}(c_{ij}^{[k]}) * (c_{ij}^{[k]})^2 \right)$$

where  $\text{sgn}$  is the signum function (which extracts the sign of a real number, i.e., assigns  $-1$  to negative numbers and  $+1$  to positive numbers),  $c^{[k]}$  is the Spearman's rank correlation matrix for treatment  $k$ ,  $n$  is the number of treatments,  $\beta$  is the soft-thresholding power which transforms the correlation values to place emphasis on large over small changes, and  $D$  is the matrix of distance metrics (Tesson et al., 2010). These analyses were conducted with a conservative soft-thresholding power of 4 (Dobson, Chaston, & Douglas, 2016; Hariharan et al., 2014), and clustering of modules using the WGCNA's `dynamicTreeCut()` function employed a recommended minimum cluster size of 100 (i.e., coexpression modules were required to contain at least 100 genes; Tesson et al., 2010) and a conservative cut height parameter of 0.98 (Dobson et al., 2016; Hariharan et al., 2014; Tesson et al., 2010). WGCNA merged putative modules whose expression profiles were highly similar, as estimated by highly correlated eigengenes ( $r = .8$ ; Dobson et al., 2016; Hariharan et al., 2014; Tesson et al., 2010). We used established permutation testing methods (Dobson et al., 2016; Hariharan et al., 2014; Tesson et al., 2010) with a 1,000 randomized data sets to verify that modules showed significant coexpression differences between treatments and eliminated modules that lacked clear support for differential coexpression.

Our use of the DiffCoEx pipeline has several subtle, but important, analysis implications. First, while it estimates Spearman rank correlations within treatments, no hypothesis testing is performed on these individual correlations. Second, the primary input to WGCNA for estimating modules is a distance matrix reflecting all of the data (in this case 31 samples), rather than individual matrices within treatments; the distance matrix across treatments directly corresponds to our biological hypothesis that microbial environments affect coexpression structure. Third, the use of permutation testing is a means to reduce the effects of sampling error in estimating the original rank correlations from leading to spurious coexpression differences: if small sample sizes and sampling error lead to differences

in rank correlations (and hence larger distance metric scores) simply by chance, that should also occur with permuted data.

## 2.6 | Module relationships with plant performance and functional enrichment

To provide preliminary insights into whether the modules identified as differentially coexpressed among microbial treatment could underlie plant performance, we regressed plant performance (aboveground biomass) to the eigengene of each plant module (i.e., the primary axis of variation in expression of all genes in the module) across the 31 samples. *p*-Values were adjusted for multiple testing (FDR < 0.05).

We also conducted a Gene Ontology enrichment analysis for modules that were differentially coexpressed with the algorithm from the topGO R-package (Alexa & Rahnenfuhrer, 2010). Annotation information was used for assigning functions in both the plant (Mt4.0v2; www.medicagogenome.org) and fungi (Tisserant et al., 2013). *p*-Values for enrichment of complete GO terms were generated using the Fisher exact test and adjusted to correct for multiple comparisons in topGo. The background for the GO analysis was the set of genes input into the computational pipeline (e.g., the 25,967 expressed plant genes were used as the background for the plant analysis). We report GO functions here by first identifying a set of the 10 most highly enriched functions for each module (i.e., the top 10 smallest *p*-values for functions) and then selecting the most significant child term within that set of 10. While there are many possible criteria for narrowing functional discussion, we used this approach within the text of this article to reduce bias while identifying important and interpretable functions. The full list of functional enrichment is included in the supplementary materials.

## 3 | RESULTS

### 3.1 | Descriptive sequencing and read-mapping statistics

A total of 753,894,804 raw reads were generated ( $\sim 24.3 \pm 0.6$  million reads per sample) with  $\sim 83.63 \pm 1.53\%$  mapping to the *M. truncatula* genome. In the M+ treatment,  $\sim 11.40 \pm 1.71\%$  of reads mapped to the mycorrhizal (*R. irregularis*) genome while very few reads mapped to the *R. irregularis* genome in the M– treatment ( $0.17\% \pm 0.01$ ), indicating that M– plants did not associate with mycorrhizal fungi (Afkhani & Stinchcombe, 2016). Sequence data are available in the NCBI Sequence Read Archive (SRP07824).

### 3.2 | Are genes nonadditively affected by multiple mutualists more highly connected?

The connectivity of genes with an additive effect ( $z = 3.1$ ,  $p = .001$ ) and nonadditive effects ( $z = 5.8$ ,  $p < .00001$ ) by multiple mutualists is higher than random expectation (Figure 1, Table S1). Interestingly,

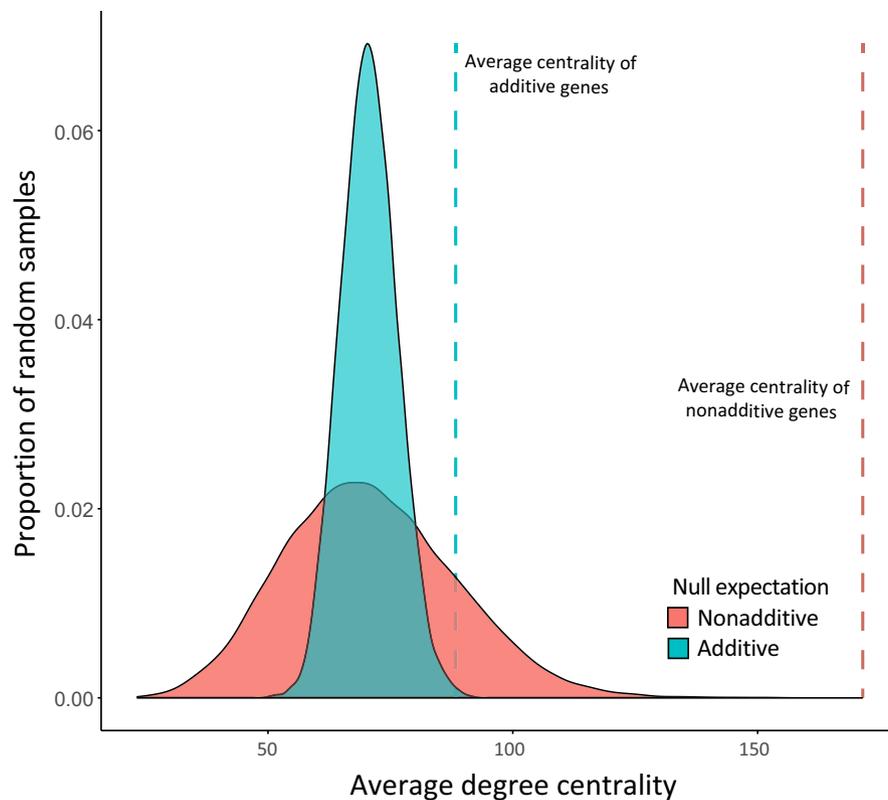
we find that genes with a nonadditive effect of multiple mutualists on expression had a nearly twofold higher mean centrality than genes that show an additive effect ( $\bar{x}_{\text{additive}} = 88$ ,  $\bar{x}_{\text{nonadd}} = 171$ ,  $p = .002$ ). These results indicate that the genes with nonadditive effects are highly connected and possibly exert on average higher influence over the molecular phenotype of the host organism than other genes in the network.

### 3.3 | Do multispecies mutualisms substantially change gene coexpression networks of hosts?

Of the 25,967 expressed plant genes that were input into our differential coexpression network analysis 17,271 genes were assigned to one of 18 differentially coexpressed modules. These modules consist of groups of coexpressed genes that differ in their correlation structures across the four microbial environments (Figures 2 and S3) and included 67% of expressed plant genes, indicating that variation in microbial environment leads to substantial variation in the coexpression patterns (Tables 1 and S2 for gene composition of each module).

Permutation analysis identified which microbial environments led to differential coexpression patterns for each of the 18 modules and allowed categorization of most modules based on how mutualists and multiple mutualists impacted coexpression relationships (Tables 1 and S3). First, we found that in five of 18 modules, coexpression of genes in the presence of both mutualists (M+R+) was significantly different from all other treatments (“Multiple Mutualist Effects”; Table 1; Figure 2), suggesting that multispecies mutualisms can cause substantial rewiring of the host plant’s coexpression network. These modules were enriched for many important functions: glycolytic process ( $p = .00033$ ; black), terpenoid metabolic process ( $p = .00018$ ; skyblue3), oxidation–reduction process ( $p = .043$ ; mediumpurple3), chromatin organization ( $p = .0023$ ; sienna3) and photosynthesis ( $p = 4.8e-11$ ; paleturquoise) (Table 1). A full list of associated functions are available in the supplementary materials (see Table S4). Interestingly, the module associated with glycolytic processes (black) was also highly enriched for the nonadditive genes reported in the centrality analysis above, with 40 of the 62 identified nonadditive genes in this module ( $p = .00001$ ). Additionally, this module contained 5 out of 8 genes annotated for ammonium transport ( $p = .00124$ ).

Five of the 18 modules had unique coexpression relationships in the presence of just mycorrhizal fungi (M+R–) compared to all other treatments (“Third-Party Effects”; Table 1; Figure 2), suggesting that the presence of mycorrhizal fungi alone alters coexpression pattern, but presence of rhizobia acts as a restorative force. These modules were also highly enriched for important functions (Tables 1 and S4), including response to oxidative stress ( $p = 5.3e-07$ ; darkorange), anion transport ( $p = .0081$ ; purple), RNA processing ( $p = .0042$ ; brown), carbon fixation ( $p = .00059$ ; grey60) and pyridine-containing compound biosynthetic process ( $p = .0074$ ; darkgreen).

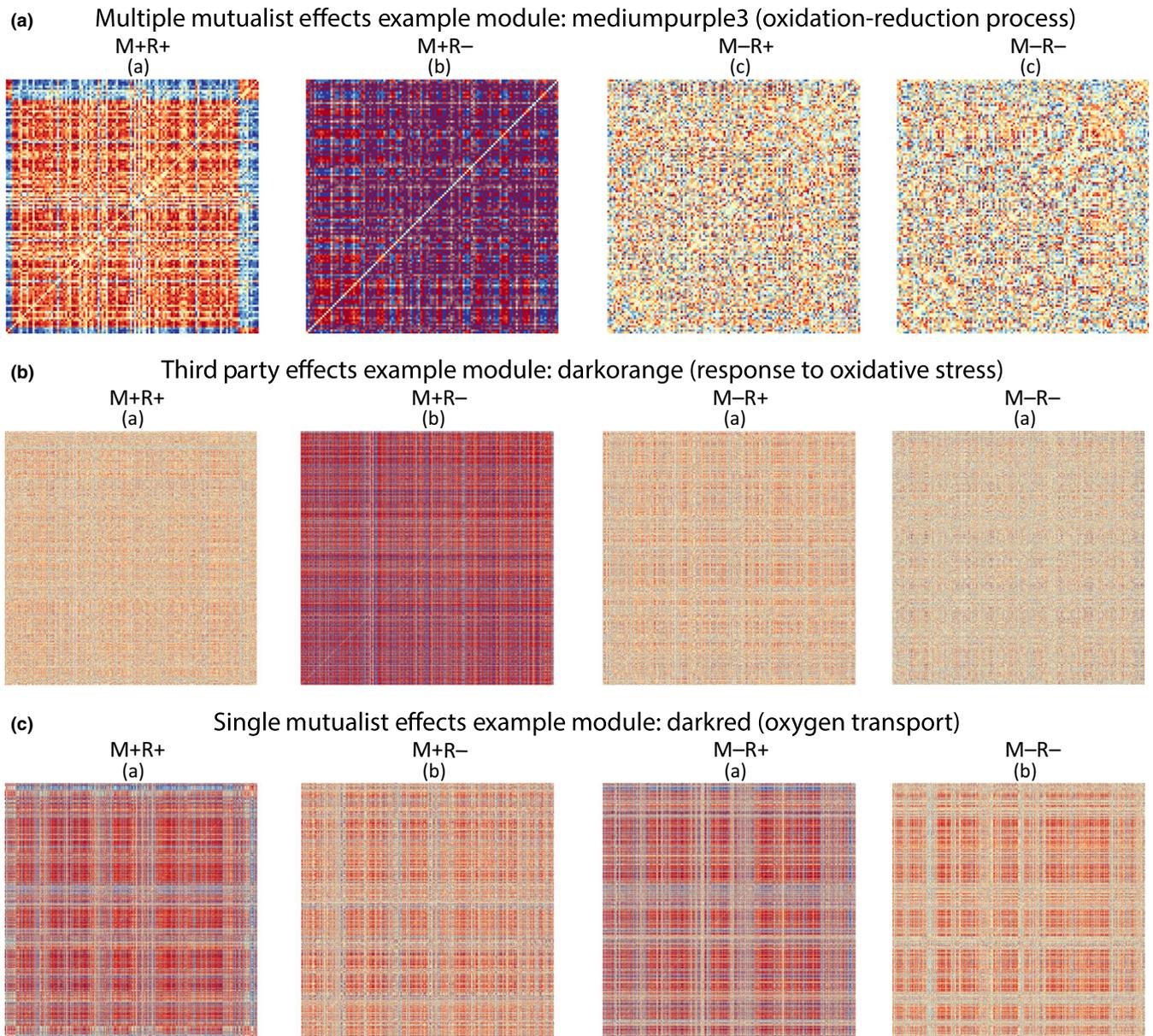


**FIGURE 1** Connectivity of genes affected by multiple mutualisms additively and nonadditively. The average centrality within the gene network of randomly selected *Medicago truncatula* genes was calculated to generate the null expectation ( $n = 10,000$ ). Two null expectations were generated to account for the difference in sample size between additive (>500 genes) and nonadditive (62 genes) gene sets. The actual centrality of additive and nonadditive genes is shown as blue and red dashed lines, respectively. The connectivity of genes with an additive effect ( $z = 3.1$ ,  $p = .001$ ) and nonadditive effects ( $z = 5.8$ ,  $p < .00001$ ) of multiple mutualists is greater than expected by chance. Further, nonadditive genes are significantly more connected than additive genes ( $p = .002$ )

We also identified one module with differential coexpression in response to any microbial environment with a mutualist (“Mutualist Effects”; Table 1). For this module, which is enriched for DNA packing ( $p = 1.6\text{e-}18$ ; module midnightblue), the mutualist treatments (M+R+, M+R– and M–R+) have similar coexpression structure which is distinct from a nonmicrobial environment (M–R–). Therefore, for this module, participation in mutualism altered the coexpression network regardless of the identity or diversity of mutualists. In addition, two modules had distinct coexpression relationships driven by a single microbial mutualist regardless of the presence of the other microbe (“Single Mutualist Effect”; Table 1; Figure 2). In the magenta module (enriched for phosphorylation;  $p = 3.4\text{e-}19$ ; Table 1), differential coexpression patterns depended on mycorrhizal fungi such that plants in mycorrhizal treatments (M+R+ and M+R–) had different coexpression relationships from those in nonmycorrhizal treatments (M–R+ and the M–R–). While the mycorrhizal fungi environment appears to be important for driving coexpression differences in this module, the rhizobia environment appeared to be important for driving coexpression differences of the darkred module. The latter, which was enriched for oxygen transport ( $p = 2.0\text{e-}12$ ; Table 1), had different coexpression of genes in the presence of rhizobia (M–R+ and M+R+) vs. in its absence (M–R–, M+R–).

### 3.4 | Do sets of host genes that are differentially coexpressed in response to microbial mutualists explain variation in plant performance?

We identified five modules whose coexpression network structure was affected by microbial mutualists that were also significantly associated with host plant performance (Figure 3). For each of these modules, the eigengene (the primary axis of variation in coexpression) was significantly correlated with plant aboveground biomass after FDR correction ( $|r|$  ranging from .50 to .54). Three of the five differentially coexpressed modules that explained performance in the host plant had coexpression that was affected by interactions among the microbial mutualists (i.e., in the multiple mutualist effects or third-party effects categories; Table 1), again suggesting that multiple mutualist-driven changes in plant performance could be caused by substantial rewiring of the host plant’s coexpression network. Another module (darkred, enriched for gas transport) that was significantly associated with plant performance had changes in coexpression that were driven predominantly by rhizobia, but also trended towards being affected by multiple mutualists ( $p$ -value between M+R+ and M–R+ was marginal:  $p = .08$ ; Table S3).



**FIGURE 2** Coexpression changes in plants among microbial environments. (a) Example heat maps of plant coexpression in a “Multiple Mutualist Effects” module in which coexpression of genes in the presence of both mutualists (M+R+) was significantly different from all other treatments. (b) Example heat maps of plant coexpression in a “Third-Party Effects” module in which coexpression with mycorrhizal fungi alone (M+R-) is unique but the presence of rhizobia (a third-party mutualist) returned coexpression structure to that observed without fungi (i.e., same as in treatments M-R+ or M-R-). (c) Example heat maps of “Single Mutualist Effects” module in which coexpression is dependent on the presence of rhizobia (i.e., M+R+ and M-R+ have different coexpression from M+R- and M-R-). The heat maps show the coexpression of plant genes in each of our four microbial treatments in an  $N \times N$  grid where  $N$  is the number of genes in the module and each pixel represents the Spearman rank correlation between the expression levels of any two genes. Red and blue indicate positive and negative correlations, respectively. M+R+ indicates coexpression of plant genes grown with mycorrhizal fungi and rhizobia, M+R- indicates plants grown with only mycorrhizal fungi, M-R+ indicates plants grown with only rhizobia, and M-R- indicates plants grown with no microbes. Significance differences in coexpression structure among microbial treatment from permutation testing of these DiffCoEx-identified modules indicated by different lowercase letters

### 3.5 | How is fungal gene coexpression affected by rhizobia?

We identified 22 fungal modules, containing 48% of the 13,247 fungal genes (Table S2), that have distinct coexpression network

structure in the treatment with both partner mutualists compared to the treatment with only mycorrhizal fungi (Table S3), suggesting a very strong effect of third-party mutualists on mycorrhizal coexpression (i.e., lots of changes in network connectivity). These modules were enriched for many important mycorrhizal metabolic and cellular

**TABLE 1** Effect of microbial environment on coexpression for host plant genes

Module ID <sup>A</sup>	Categorization <sup>B</sup>	Permutation test results <sup>C</sup>				<i>p</i> -Value for plant performance <sup>D</sup>	Module's functional enrichment <sup>E</sup>
		M+R+	M+R–	M–R+	M–R–		
Paleturquoise	Multiple Mutualist Effects	a	b	c	c	<b>.01</b>	Photosynthesis
Sienna3	Multiple Mutualist Effects	a	b	c	c	<b>.01</b>	Chromatin organization
Skyblue3	Multiple Mutualist Effects	a	b	b	b	.12	Terpenoid metabolic process
Mediumpurple3	Multiple Mutualist Effects	a	b	c	c	.14	Oxidation–reduction process
Black	Multiple Mutualist Effects	a	b	c	c	.37	Glycolytic process
Brown	Third-Party Effects	a	b	a	a	<b>.01</b>	RNA processing
Grey60	Third-Party Effects	a	b	a	a	.11	Carbon fixation
Darkgreen	Third-Party Effects	a	b	a	a	.22	Pyridine-containing compound biosynthetic process
Purple	Third-Party Effects	a	b	a	a	.71	Anion transport
Darkorange	Third-Party Effects	a	b	a	a	.74	Response to oxidative stress
Midnightblue	Mutualist Effects	a	a	a	b	.32	DNA packaging
Darkred	Single Mutualist Effects	a	b	c	b	<b>.01</b>	Oxygen transport
Magenta	Single Mutualist Effects	a	a	b	c	.51	Phosphorylation
Red	Other	a	b	ad	cd	<b>.01</b>	Regulation of primary metabolic process
Cyan	Other	a	b	b	ab	.11	Defence response
Green	Other	a	ac	bc	a	.32	Regulation of transcription, DNA-templated
Darkolivegreen	Other	a	b	c	a	.39	Putrescine metabolic process
Blue	Other	a	bc	ac	ac	.57	Translation

<sup>A</sup>Modules identified in the differential coexpression network analyses are given individual and unique colour names. <sup>B</sup>The modules were placed into five categories based on their differential expression among microbial environments determined via <sup>C</sup>permutation testing in DiffCoEx computational pipeline. Significant differences ( $p \leq .05$ ) among microbial treatments indicated by distinct lowercase letters. <sup>D</sup>Significant associations between the primary axis of variation in the coexpression of modules and plant performance (shoot mass) are indicated as bolded *p*-values. Analyses were FDR-corrected. <sup>E</sup>Enrichment analyses were used to identify key functions associated with each differentially coexpressed module. A full list of associated function are available in the supplementary materials, but we have listed the most specific function (i.e., child term) from the top ten most significant functions associated with each module.

processes (Table S4), including phosphorylation (Myco\_darkslateblue,  $p = .0065$ ), carboxylic acid transport (Myco\_darkturquoise,  $p = .0046$ ), lipid glycosylation (Myco\_pink,  $p = .00086$ ) and *N*-acetylglucosamine metabolic process (Myco\_saddlebrown,  $p = .02$ ). Full enrichment lists for all 22 fungal modules that were significantly differentially coexpressed in response to rhizobia are available in Table S4.

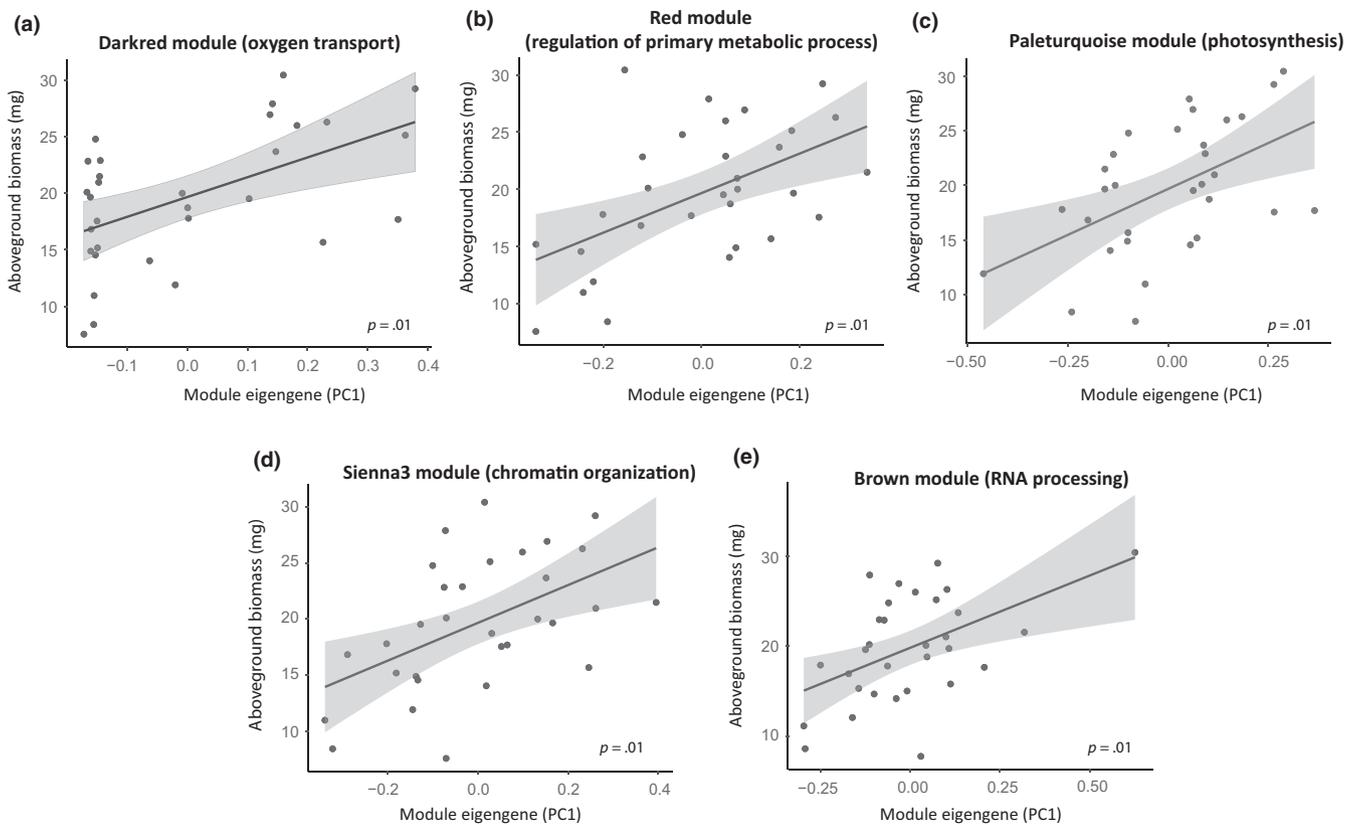
### 3.6 | How is the joint coexpression of plant and fungal genes affected by the presence of rhizobia?

To examine how rhizobia could alter coordination in gene expression between host plants and their mycorrhizal fungi symbionts, we identified “across-symbiosis” gene modules (i.e., modules containing both mycorrhizal fungi and plant genes expressed together; Table S2) that are differentially coexpressed based on the presence or absence of rhizobia. We detected 18 modules with significant differences in coexpression structure in response to rhizobia; these modules were on average composed of  $63 \pm 3\%$  plant genes (and  $37 \pm 3\%$  fungal genes) and were enriched for important functions (Tables S3–S4). For example, module Sym\_salmon1 was enriched for lipid transport

in the plant ( $p = .0030$ ) and for potassium ion transport in the mycorrhizal fungi ( $p = .0067$ ). Additionally, module Sym\_thistle3 was enriched for regulation of metabolic process in the plant ( $p = .0124$ ) and protein phosphorylation in the mycorrhizal fungi ( $p = .0015$ ). Full enrichment lists for both fungal and plant gene sets for each of the 18 modules are available in Table S4.

## 4 | DISCUSSION

Complex community interactions between multiple mutualists are important for both the ecology and evolution of participating species (Afkhami et al., 2014; Harcombe, Betts, Shapiro, & Marx, 2016; Palmer et al., 2010). Many of these interactions are microscopic, take place below ground and involve resource exchange that can be difficult if not impossible to observe (Burghardt et al., 2017). A transcriptomic approach offers the prospect of identifying hundreds to thousands of molecular phenotypes associated with these interactions. Our gene network analysis of coexpression patterns in response to multiple mutualists revealed three major findings. First, individual genes whose expression is affected by multiple mutualists



**FIGURE 3** Relationship between plant performance and differentially coexpressed genes. Five differentially coexpressed modules of genes explain variation in aboveground biomass. These modules were annotated for oxygen transport (a), regulation of metabolic processes (b), photosynthesis (c), chromatin organization (d) and RNA processing (e). X-axis is the eigengene, or the primary axis of variation in expression across the gene set (scaled with mean = 0,  $SD = 1$ ), and the y-axis is the plant aboveground biomass. Each point is an individual plant, grey regions indicate 95% CIs, and listed  $p$ -values are FDR-corrected

in nonadditive ways appear to be more connected and potentially more important for coexpression networks than a random set of genes. Second, interacting with multiple mutualists leads to changes in gene coexpression network structure, including changes to coexpression of modules that are associated with plant performance. Finally, one mutualist, rhizobia, affected gene coexpression of an alternate mutualist (fungi) and plants and fungi simultaneously. We consider these results in turn below.

#### 4.1 | Are you connected? Network analysis of nonadditively expressed genes

There are competing hypotheses for the network position of genes whose expression is nonadditively affected by multiple mutualists. On the one hand, these genes may be central to nonadditive transcriptomic and performance responses to multiple mutualists and are thus central in gene networks. Nonadditive changes in expression in these genes may thus affect many other gene networks and pathways, potentially underlying synergistic effects on performance and fitness of interacting with multiple mutualists. On the other hand, it may be that these genes, which respond to biological cues (the presence and absence of two other species) that are variable in space

and time, are peripheral in gene networks. Under this interpretation, genes whose expression affects growth, physiology, nutrient acquisition and allocation, and performance in response to symbiosis may be less connected because of their potential widespread pleiotropic effects.

Our results clearly indicate that nonadditive genes appear to be at the centre of gene coexpression networks and are more connected than both a random sample and genes whose expression is affected by multiple mutualists in additive fashion. If we use connectivity as a proxy for biological importance—how tightly associated with transcription of many other genes throughout the genome and their potential for relaying information in networks—these results suggest that a few dozen genes ( $N = 62$ ) may be central players in plastic responses to multiple mutualists.

Many studies of gene network expression in ecology and evolution have focused on evolutionary components of gene networks. For example, there is a large literature on how network properties affect evolutionary divergence of genes between species (Carlson et al., 2006; Jordan, Mariño-Ramírez, Wolf, & Koonin, 2004; Jovelín & Phillips, 2009) and polymorphism within species (Josephs et al., 2017; Mähler et al., 2017). A common underlying hypothesis to many of these studies is that genes with more connectivity or

involved in more biological processes are likely to show higher pleiotropy (He & Zhang, 2006). However, we have much less evidence on plastic responses of gene networks to ecological cues such as the presence or absence of other species.

Recent work by Des Marais et al. (2017) in *Arabidopsis thaliana* provides an interesting point of comparison. They found that genes showing Genotype  $\times$  Environment interactions for expression (eG  $\times$  E) in response to drought were significantly more peripheral in gene expression networks, while those showing eG  $\times$  E in response to cold were significantly more central in networks. They suggested that because nearly all genotypes of *Arabidopsis* will experience some sort of drought throughout some part of their life cycle it could lead to many potential, fine-tuned ways to adjust phenotypes and expression in response to drought. In contrast, because only some genotypes will experience extreme cold, it may be that there are fewer adaptive phenotypes, requiring coordinated changes in expression across many genes. In our experiment, nonadditively expressed genes are similar to the cold-responsive eG  $\times$  E genes studied by Des Marais (i.e., we observed genes showing Fungi  $\times$  Rhizobia interactions for expression; both sets of genes are at the centre of networks). If their general interpretation is correct, it suggests in our system there might only be a few ways to coordinate multispecies mutualisms and that nonadditively expressed genes are central to this multispecies response. Pairwise or single-mutualism studies may fail to identify centrally important genes for coexpression.

## 4.2 | It takes a network: Understanding multispecies mutualisms impacts on host coexpression

Moving beyond a “gene at a time” approach provided new insights into hosts’ molecular response to multispecies mutualists. First, we noted that the mutualisms with rhizobia and mycorrhizal fungi resulted in substantial changes in the coexpression network topology of host plants, such that >67% of plant genes were in modules that had significant changes in coexpression in response to microbial mutualisms. In particular, the *interaction* between these partner mutualists had important consequences for coexpression. For instance, ~30% of the identified modules had distinct coexpression structure in the presence of multiple mutualists, suggesting simultaneous interactions with multiple partners can cause significant rewiring of host plants expression networks. These modules were enriched for a variety of important and intriguing functions. For example, the multiple mutualist-affected “skyblue3” module was strongly associated with terpenoid metabolic processes, suggesting that interactions with multiple mutualists may alter production of chemicals important for plant defence against herbivores, and communication between plants and other types of mutualists (Gershenson & Dudareva, 2007). Further, an additional ~30% of identified modules had unique coexpression structure that occurred when grown with only mycorrhizal fungi. Interestingly, in these third-party effects modules, rhizobial presence returned coexpression structure

to that observed without mycorrhizal fungi (i.e., same as in treatments M–R+ or M–R–). Therefore, rhizobia may have overwhelmed the coexpression changes generated by the fungi, restoring network structure to that found in other microbial environments. While we previously identified a potentially important, but also small, set of genes that were differentially expressed in response to multiple mutualists (Afkhami & Stinchcombe, 2016), our new differential coexpression analysis revealed substantial rewiring of gene expression interactions across the host plant genome.

Second, we found that most of the groups of coexpressed genes that significantly explained plant performance showed either multiple mutualist effects or third-party effects on their coexpression. Our finding indicates that interactions among multiple microbial mutualists that drive significant changes in coexpression may be important for plant performance and underlie some of the synergistic fitness effects observed in tripartite mutualisms. In fact, many of the performance-associated modules with unique coexpression in the presence of multiple mutualists were enriched for functions such as photosynthesis, which is likely important for plant fitness and carbon-based rewards provided to microbes (Afkhami & Stinchcombe, 2016; Larimer et al., 2014; Ossler et al., 2015). Another performance-associated module was enriched for oxygen transport (containing 11 out of the 16 *M. truncatula* genes annotated for oxygen transport). Oxygen transport genes, such as leghemoglobin genes, are known to be necessary for the maintenance of nitrogen-fixing nodules (Ott et al., 2005) and can also be induced by colonization of arbuscular mycorrhizal fungi (Frühling, Roussel, Gianinazzi-Pearson, Pühler, & Perlick, 1997; Vieweg et al., 2004). This module trends towards coexpression being affected interactively by multiple mutualists (Table S3), indicating future study into interactive effects of these mutualists on oxygen transport may be informative. While significant associations between performance and coexpression suggest that changes in plant coexpression led to changes in plant performance, these analyses are correlative and thus cannot fully establish causality. Therefore, we advocate for future work (i) manipulating growth environment and plant genotype to determine whether modules’ relationships with performance shift with these sources of variation and (ii) directly manipulating modules (e.g., knocking out central “hub” genes) to test for changes in performance phenotypes.

Third, we found that a surprising 40 of the 62 genes identified as having expression nonadditively affected by multiple mutualists [in a gene-by-gene differential expression analysis in Afkhami and Stinchcombe (2016)] were part of a single module (black, enriched for glycolytic processes). This module had a unique coexpression network structure in the presence of multiple mutualists and was significantly enriched for glycolytic processes and ammonium transport aligning with known biology of nutrient transport between host plant, mycorrhizal fungi and rhizobia (Govindarajulu et al., 2005; Udvardi & Poole, 2013). Taken with results from the degree centrality analysis, we hypothesize that a small candidate set of nonadditive genes may play important roles in coordinating responses to multispecies microbial mutualisms through the trade of resources.

### 4.3 | Party crashers and Cross-talk: Third-party mutualists and microbial coexpression across the symbiosis boundary

A key aspect to unravelling how organisms regulate interactions in complex communities is to investigate multispecies mutualisms from the perspective of each partner species and the across-symbiosis interaction. Our analyses provided some important preliminary insights for this goal and highlight important future directions. One surprising result from our examination of coexpression across the mycorrhizal genome was that rhizobia caused significant changes in coexpression structure with approximately half of expressed mycorrhizal genes in one of 22 differentially coexpressed fungal modules. These results indicate that a third-party mutualist can cause major rewiring of another microbial partner's molecular network, potentially inducing or suppressing molecular and biochemical pathways that underlie important functions, including many fundamental cellular processes of the mycorrhizal fungi.

We noted that these molecular changes in fungi may result from one or, more likely, a variety of components of the interaction with rhizobia, including both direct and indirect effects. For example, by increasing availability of N through fixation, rhizobia could enhance host plant performance (Afkhani et al., 2014; Larimer et al., 2014; Ossler et al., 2015) *indirectly* leading to inducement or suppression of molecular processes in mycorrhizal fungi such as colonization or growth. Rhizobia and mycorrhizal fungi could also have *direct* positive or negative effects on each other's fitness, and associated changes in molecular processes, as they both occur in plant roots and a number of studies have shown evidence for colocalization (with mycorrhizal colonization of up to ~75% of the nodules; Scheublin & van der Heijden, 2006). Close proximity of symbionts to one another could facilitate easier transfer of resources or direct inhibition among symbionts. Scheublin and van der Heijden (2006) found that fungal-colonized nodules typically did not fix atmospheric nitrogen, which could mean that fungal colonization of nodules leads to a negative direct interaction among microbial partner species. While differentiating between direct and indirect transcriptional effects of mycorrhizal fungi and rhizobia on each other is challenging and beyond the scope of this paper, future work using split-root design experiments (Batstone, Dutton, Wang, Yang, & Frederickson, 2017) to spatially separate microbes could eliminate direct interactions and allow decoupling of direct and indirect effects.

Finally, many species depend on nutritional resources acquired through resource exchange mutualisms (Boucher, James, & Keeler, 1982; Hosokawa, Koga, Kikuchi, Meng, & Fukatsu, 2010; Ji & Bever, 2016). Recent work, for instance, has increasingly documented extensive metabolic cooperation for essential amino acid synthesis in tightly coevolved insect-microbe symbioses (Ankrah, Luan, & Douglas, 2017; Wilson & Duncan, 2015) and within microbiomes, allowing microbial communities to utilize substrates that would otherwise be inaccessible (Seth & Taga, 2014). Beyond production of resources, another important aspect of resource exchange is transporting of rewards between partners. Our results from an across-symbiosis analysis show that transport is enriched for plants and mycorrhizal

fungi in modules that are differentially coexpressed when rhizobia is present (the *sym\_chocolate3* and *sym\_salmon1* modules), suggesting that this third-party mutualist may shift trade dynamics between the other two species. By identifying groups of genes in plants that are coexpressed with genes in the mycorrhizal genome, our coexpression network approach provides initial insights into across-symbiosis molecular interactions and candidate modules for future exploration.

## 5 | CONCLUSION AND FUTURE DIRECTIONS

Our study demonstrated that multispecies mutualisms have substantial effects on the complex molecular network of gene expression interactions in host plants, microbes and across symbiotic boundaries. Although challenging to incorporate both ecological complexity (i.e., moving beyond pairwise interaction studies) and molecular complexity (e.g., moving beyond a "gene at a time" approach), we detected important consequences for the molecular phenotypes of host organisms and key candidate genes for breeding and plastic responses to ecological variation. In our opinion, three general classes or types of experiments are likely to be profitable going forward. First, studies that expand the range of genotypes and environmental conditions (e.g., C-, N-, P-limited or supplemented conditions) will be necessary to fully integrate ecological and genetic complexity in studies of multispecies mutualisms. Second, studies manipulating gene function and/or utilizing gene validation approaches are important next steps for improving our understanding of causality as well as which molecular changes are most important for performance within identified candidate modules. For example, future work directly manipulating hub genes within the performance-associated modules and measuring host transcriptional and performance responses could provide valuable mechanistic and causal insight. Third, complementary studies delving more deeply into the effect of microbial mutualists on each other, such as using split-root design experiments to distinguish between direct and indirect transcriptional effects of partner species on one another, are also needed. Collectively, studies like these will allow us to better understand the mechanistic basis as well as ecological and evolutionary consequences of multispecies mutualisms.

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## DATA ACCESSIBILITY

Phenotypic data are available through Dryad (<https://doi.org/10.5061/dryad.8f66s>); expression data are available at NCBI's Sequence

Read Archive (SRP078249). All annotated R scripts for the differential coexpression network analyses and the normalized expression data is also available through Dryad (<https://doi.org/10.5061/dryad.2hj343f>).

## AUTHOR CONTRIBUTIONS

All authors discussed the results and contributed to writing the final manuscript and revisions. S.X.P. conducted all bioinformatics processing, network analyses, and other data analysis and created tables, figures and network resources. J.R.S. made substantial contributions to analysis/experiment, conceptual framing, experimental design and interpretation. M.E.A. conceptualized the study, conducted the experiment, made substantial contributions to analysis decisions and interpreting results and oversaw the bioinformatics project and organization of manuscript.

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## SUPPORTING INFORMATION

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