CHAPTER 12.11.1

Synergism and symbioses: unpacking complex mutualistic species interactions using transcriptomic approaches

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12.11.1.1 The importance of multispecies mutualisms

Mutualisms, interactions that benefit participating species, are ubiquitous in every habitat and kingdom (Bronstein 1994; Palmer et al. 2010) and function as central players in both evolutionary and ecological processes. For example, they undergird major events in the history of life on Earth, such as the origin of eukaryotic cells (Margulis 1970), plants' invasion of land (Pirozynski and Malloch 1975), and adaptive radiations (Weber and Agrawal 2014), as well as ecological processes, such as the maintenance of biodiversity (Bascompte and Jordano 2007; Fontaine et al. 2006) and succession (Rudgers et al. 2007).

Much of the early (and current) studies of mutualism focus on highly coevolved, pairwise interactions between two partner species (e.g. *Acacia* ant–*Acacia* interaction; Janzen 1966) or interactions among a small number of functionally similar mutualists (e.g. a plant with its insect pollinators; Bascompte and Jordano 2007; Brown et al. 2002; Lau and Galloway 2004). Yet, in reality, many organisms interact with numerous different and functionally distinct mutualistic partners throughout their lifetimes (Afkhami et al. 2014). In terrestrial habitats, for instance, plants may depend on pollinators to reproduce (Kearns et al. 1998), seed dispersers to reduce competition with parental plants (Higashi et al. 1989), and endosymbionts for increased nutrient and water intake (Bowles et al. 2016; Gustafson and Casper 2006). The effects from these complex multispecies mutualisms play key roles in local diversity, community composition, and even ecosystem functioning that cannot be detected from pairwise studies (Afkhami et al. 2014; Palmer et al. 2010; van der Heijden et al. 2008). Further, mutualism diversity could help maintain communities and protect against environmental change (Bascompte and Jordano 2007; Fontaine et al. 2006).

Although a community perspective on mutualisms has developed predominantly in the past few decades (Bascompte and Jordano 2007; Stanton 2003), a theoretical framework and empirical studies on multiple mutualisms have already demonstrated some unexpected outcomes (Afkhami et al. 2014; Stanton 2003). In particular, multiple mutualisms can have nonadditive effects on the performance of their shared partner, including many examples of synergistic effects (Abd-Alla et al. 2014; Jia et al. 2004; Larimer et al. 2014; McKeon et al. 2012; Oliveira et al. 2005; Palmer et al. 2010; Stachowicz and Whitlatch 2005). For example, in highly alkaline sediment, dual inoculation of both nitrogen-fixing bacteria (*Frankia* spp.) and arbuscular mycorrhizal fungi (*Glomus intraradices*) had synergistic effects on growth and nutrition of European Alder (*Alnus glutinosa;* Oliveira et al. 2005). Additionally, these nonadditive effects may help explain the maintenance of multiple mutualists and endosymbionts, where some partners are parasitic or confer no benefit in isolation, but are beneficial in combination with other mutualists (Gustafson and Casper 2006; Palmer et al. 2010). Elucidating the performance consequences of multiple mutualistic effects and their mechanistic basis is a critical step in advancing our understanding of the role of these interactions within communities and ecosystems.

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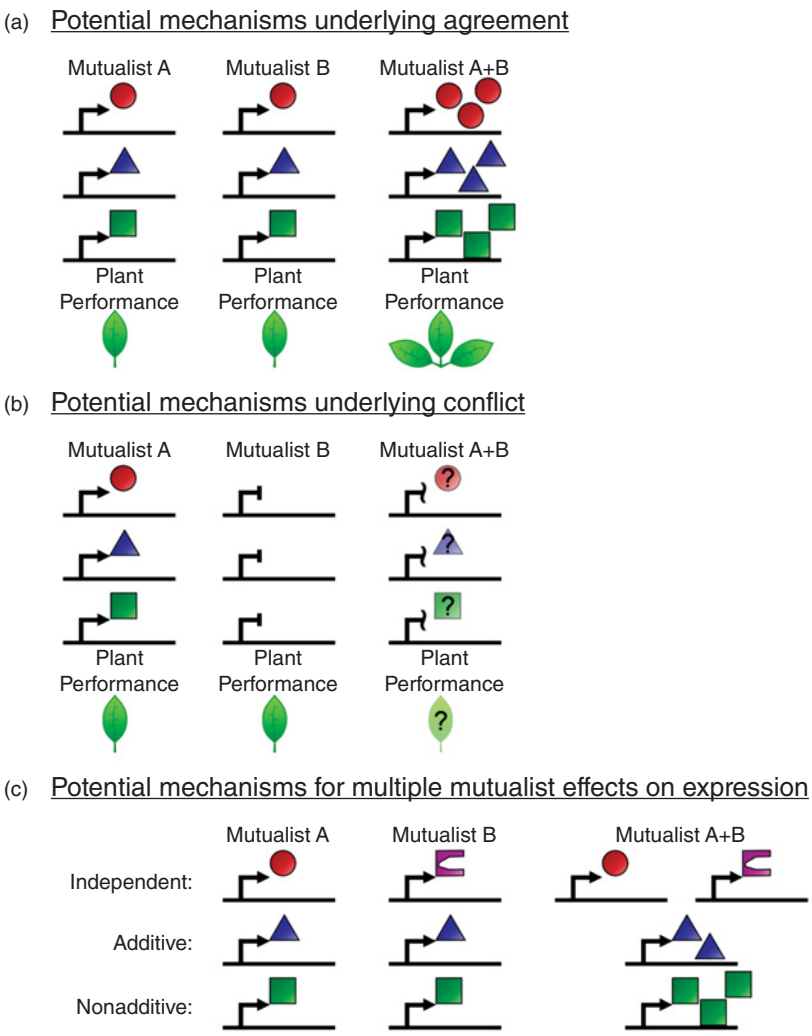

Figure 12.11.1.1 Schematic of potential mechanisms by which multispecies mutualisms may act on expression and plant performance. Each shape represents a different gene expression product and number of leaf shapes indicates relative performance of a host plant. Upregulation is indicated by an arrow head, downregulation or inhibition of expression by a bar, and unknown effects on expression by a "~." (a) Agreement over regulation – mutualists A and B are in agreement over the direction of regulation for three genes; both mutualists individually upregulate gene expression, resulting in increased gene products and performance of the plant. (b) Conflict over regulation – mutualists A and B have positive effects on plant performance in pairwise relationships and act on the same genes; however, they have opposing effects on gene expression. As a result, when grown together, mutualists may conflict over the direction of regulation and the consequences for plant performance are difficult to predict. (c) Schematic of the diverse effects multispecies mutualism may have on expression. Independent: each mutualist may act via independent genes and pathways without affecting the other mutualist (e.g. mutualist A regulates genes 1 while mutualist B regulates gene 2). Additive: affected genes and pathways may be shared between mutualists such that when organisms are grown with multiple mutualists the expression level can be determined by summing the effects when grown with mutualist A and B alone (i.e. effect of mutualist A + effect of mutualist B = effect of multiple mutualists). Nonadditive: Interactive effects among multiple mutualists may result in nonadditive gene expression, in which effects of multiple mutualists on regulation cannot be determined by effects of single mutualists on regulation (i.e. effect of mutualist A + effect of mutualist B ≠ effect of multiple mutualists).

12.11.1.2 Regulation of genetic pathways in multispecies mutualisms

While studies of mutualist performance provide information on biological outcomes, a deeper understanding of the genetic pathways and their regulation in multispecies mutualism can provide mechanistic models for shared pathways of understudied multiple mutualist interactions. Much like fitness effects of multispecies mutualisms, these partner species may "conflict" (Figure 12.11.1.1b) over or "cooperate" (Figure 12.11.1.1a) in the regulation of their shared host's gene expression. In isolation, the presence of one mutualist may lead to increased expression while the presence of another leads to decreased expression. For example, the presence of an ant defender may cause upregulation of plant genetic pathways associated with production of extra-floral nectar while the presence of pollinators may lead to downregulation of these genes to reinvest resources in floral nectar. These partner mutualists are potentially in conflict over the regulation of extra-floral nectar pathways, making the outcome for gene regulation and plant fitness unclear (Figure 12.11.1.1b) when both mutualists are present. Alternatively, two mutualists might each induce the upregulation of similar pathways – for example, both ants and pollinators may induce upregulation of molecular pathways underlying carbon metabolism. In this case, carbon is a resource that both partners use and can "agree" on its regulation – i.e. how the fitness of each mutualist partner responds to the reward is in the same direction (Figure 12.11.1.1a). Further, whether in agreement or conflict over the direction of regulation, multiple mutualists may jointly regulate genes in additive or nonadditive ways (Figure 12.11.1.1c), making the magnitude of the outcomes difficult to predict. It is also possible that mutualists may regulate unique pathways and thus different sets of genes (Figure 12.11.1.1c). Detailed molecular studies are needed to ascertain how complex interactions impact the molecular processes underpinning the fitness effects of multiple mutualists.

12.11.1.3 *Medicago truncatula* as a model system for the molecular basis of multiple mutualist effects

Medicago truncatula is an excellent system to explore multispecies mutualisms for several reasons. First, *M. truncatula* participates in a tripartite interaction with two critically-important and common endosymbionts, rhizobia and mycorrhizal fungi (Schenk et al. 2012). Second, there are substantial genomic resources available for *M. truncatula* and several of its partner species (e.g. rhizobia *Ensifer meliloti* and *Ensifer medicae* and mycorrhizal fungi *Rhizophagus intraradices* and *Glomus mosseae*), making studies of the molecular basis of these complex interactions possible (Reeve et al. 2010, Terpolilli et al. 2013; Tisserant et al. 2013; Young et al. 2011). Transcriptomic studies on pairwise mutualistic interactions (e.g. *M. truncatula*–mycorrhizal fungi or *M. truncatula*–rhizobia) have provided important insights on mutualistic partner effects on plant gene expression (e.g. see Sections 12.11.1.6 and 12.11.1.7).

In this chapter, we discuss our recent ecological and molecular studies of *M. truncatula* that extend the understanding of how plants respond to endosymbionts by considering the multispecies mutualism contexts. We explore and expand upon the work from Afkhami and Stinchcombe (2016), where a factorial experiment compared genome-wide expression of *M. truncatula* roots grown with *R. irregularis* (arbuscular mycorrhizal fungi), *E. meliloti* (rhizobium), both symbionts, or neither. We first discuss the system-wide effects of symbionts alone and together on plant performance (see Section 12.11.1.4) and genome-wide effects on expression (see Section 12.11.1.5). Additionally, we describe and categorize gene-level effects of multispecies mutualistic interactions, focusing on differences in independent, additive, and nonadditive outcomes (see Section 12.11.1.6) as well as the consequences for the shared Common Symbiosis Signaling Pathway (see Section 12.11.1.7; see Chapter 8.1 and Figure 8.1). While we focus on the insights yielded from the study in Afkhami and Stinchcombe (2016), we acknowledge that this is but one study (with a unique constellation of genotypes, experimental conditions, transcriptome sampling dates, etc.). Determining the generality of our findings – across genotypes, experimental conditions, or even legume species – will clearly require much additional experimental work. Thus, we conclude this chapter with a few key directions for future investigation (see Section 12.11.1.8).

12.11.1.4 Multiple mutualist effects on partner performance

Rhizobia and mycorrhizal fungi have been shown to improve plant performance in pairwise studies under many conditions (Friesen 2012; Hoeksema et al. 2010). However, since legumes (the third largest plant family) often interact with both partners simultaneously (Larimer et al. 2010; Ren et al. 2016), it is worth considering the impact of the tripartite association for plant fitness. Positive effects may result from complementarity of microbially-provided rewards; e.g. rhizobia provide plants with fixed nitrogen and mycorrhizal fungi provide water or phosphorus uptake, all of which are needed in a stressful growing environment. However, because both partners receive photosynthates as a primary reward from the host plant, they may compete for the limiting resource with cascading negative effects for the plant (Afkhami et al. 2014). Determining if the interactive effects of these two mutualists influence plant performance requires factorial manipulations (growing plants in the presence of both mutualists, neither, rhizobia alone, and mycorrhizal fungi alone) and measuring performance. While most ecological studies have focused on pairwise interactions, a smaller, but informative, set of studies in a variety of legumes has shown that these mutualists can jointly impact plant performance, ranging from synergistic positive effects to negative consequences (Larimer et al. 2010, 2014; Meng et al. 2015; Oliveira et al. 2017; Ren et al. 2016).

In Afkhami and Stinchcombe (2016), we examined for the first time the tripartite effect of these symbioses in a genome-enabled system (*M. truncatula*) on participant performances using a factorial experiment. Below we summarize the main take home messages on what is now known about the tripartite effect on plant and symbiont performances in this system:

Take home 1: rhizobia and mycorrhizal fungi had synergistic effects on *M. truncatula* performance. Plants with both partners had more leaves (Figure 12.11.1.2a), aboveground biomass (Figure 12.11.1.2b), and branches compared to plants with one or no mutualistic partner.

Take home 2: mycorrhizal fungi's presence increased rhizobial performance. When mycorrhizal fungi were present (M+R+ plants), nodulation was about 58% greater (compared to M–R+ plants; Figure 12.11.1.2c). We also note that nodulation only occurred in the roots of plants inoculated with rhizobia, suggesting that microbial contamination is unlikely.

Take home 3: rhizobia's presence increased mycorrhizal colonization and/or expression. Plants inoculated with both mutualists had a significantly higher percentage of transcript reads map to the fungal genome compared to plants inoculated with only fungi (i.e. without rhizobia; Figure 12.11.1.2d). The increased number of mycorrhizal transcripts in the presence of rhizobia suggests that rhizobia either increases abundance or gene expression of mycorrhizal fungi, or possibly both. We also note that 11.23% percent of reads mapped to the mycorrhizal genome for the plants that were inoculated with mycorrhizal fungi while only 0.17% of reads mapped to the fungal genome in the uninoculated plants, again suggesting that microbial contamination is unlikely.

12.11.1.5 Multispecies mutualism has pervasive genome-wide effects on expression

Taking a broad-scale perspective, principal component analysis (PCA) summarized the transcriptome profile of plants from

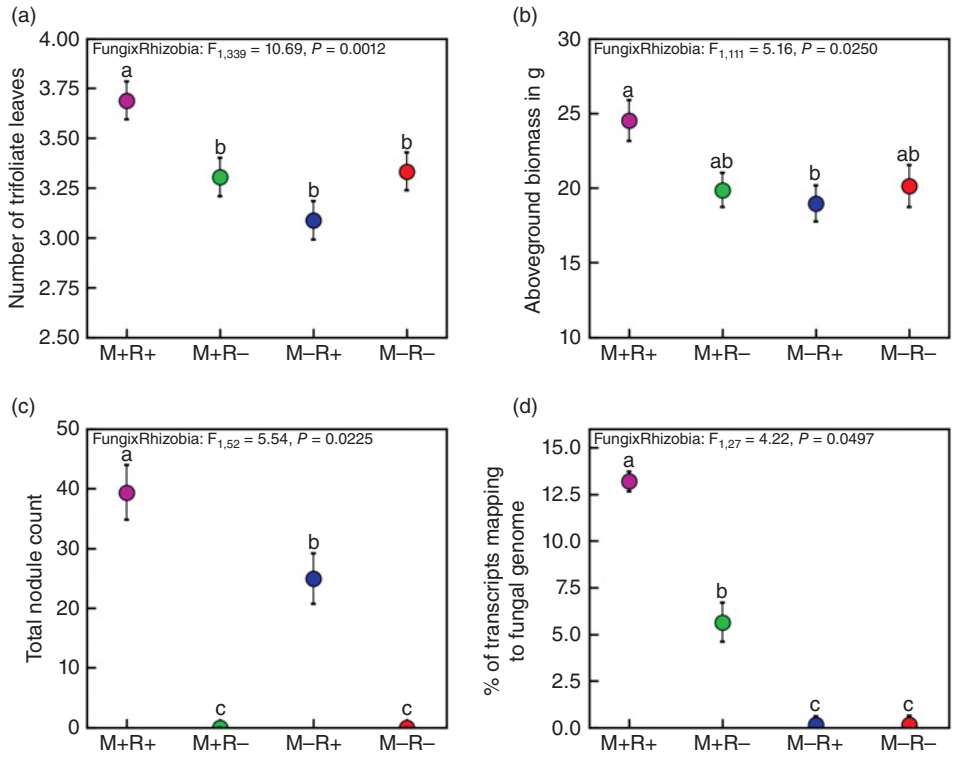

Figure 12.11.1.2 Effect of microbial treatments on performance parameters of all partners in tripartite relationship. (a,b) M+R+ treated plants showed significantly (or marginally significantly) more trifoliate leaves and aboveground biomass compared to other treatments. (c,d) Multispecies mutualism also had a synergistic effect on nodule count (a rhizobia performance parameter) and the number of mycorrhizal fungi transcripts (a possible indicator of mycorrhizal abundance).

each microbial treatment across two axes that explain 61% of the variation in genome-wide expression. This analysis suggested three key take home messages about the impact of rhizobia and mycorrhizal fungi on *M. truncatula* expression:

Take home 1: the tripartite mutualism between rhizobia, mycorrhizal fungi, and *M. truncatula* has substantial impacts on genome-wide expression. The PCA of plant gene expression showed that the mutualist environment a plant experiences (with no microbes, just fungi, just rhizobia, or both) strongly influences the overall transcriptome profile. We observed large transcriptional shifts in pairwise and tripartite plants compared to the control group (Figure 12.11.1.3a).

Take home 2: the transcriptional profile of the plant can explain variation in plant performance. Both of the first two principal component axes were significantly correlated with shoot biomass, suggesting that the overall change in gene expression may underpin changes in plant performance (Afkhami and Stinchcombe 2016).

Take home 3: genome-wide synergism suggests that multiple mutualism may have subtle, hard to detect effects on seemingly independently regulated genes. As described in Section 12.11.1.4, both rhizobia and mycorrhizal fungi likely increase the abundance of the other mutualistic partner. These results suggest that plant gene expression in response to each mutualist should be enhanced when plants are grown with both partners compared to grown with a single partner (assuming that increased abundance of mutualists lead to increased host expression). However, while closer analysis of the PCA data appears to indicate that rhizobia enhanced shifts in *M. truncatula*'s gene expression in response to mycorrhizal fungi, the reverse was not true (i.e. fungi did not cause shifts in plant expression associated with rhizobia). The asymmetry of these results may be due to ontogenetic differences in the timing of nodulation and mycorrhizal colonization. For example, extensive nodulation could occur before the time point studied whereas mycorrhizal colonization may be an ongoing process; therefore, synergistic shifts in host expression associated with rhizobia may not be detected in this study.

To reach these conclusions, we first established whether shifts in PC1 and PC2 space from the typical expression profile of control plants (represented by the center of the M–R– treatment plants in PC space) to that of the M+R– and M–R+ treatment plants likely represent expression of genes regulated by a single mutualist (i.e. "independent" genes in the gene-by-gene analysis described below in Section 12.11.1.6). We noted that expression profiles of the single mutualist treatment plants (i.e. M+R– and M–R+ plants) were very tightly and linearly displaced from that of the centroid of the control group; therefore, we calculated regression lines through each of the single mutualist treatments and the control group center (regressing PC2 on PC1,

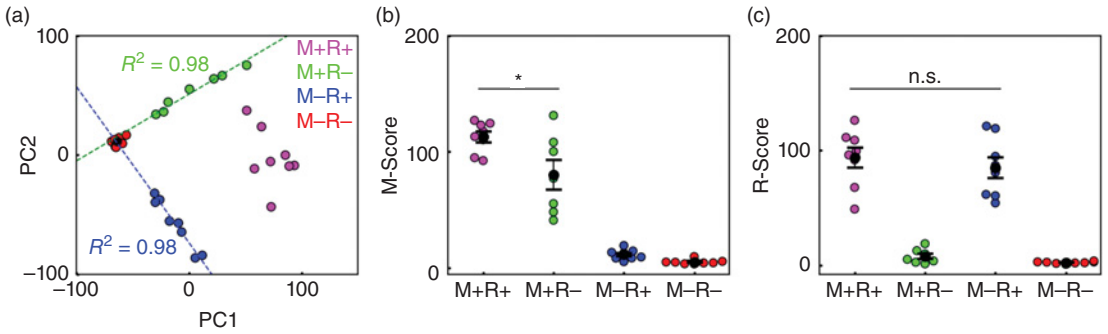

Figure 12.11.1.3 Multispecies mutualism has system-wide effects on host expression. (a) Principal component analysis of *M. truncatula* transcriptomes for the four mutualist treatments. Each point indicates the transcriptomic profile of an individual plant and clustering indicates similarity in transcriptome profile. Each color represents a different mutualist treatment with both mutualists (M+R+) in magenta, just mycorrhizal fungi (M+R–) in green, just rhizobia (M–R+) in blue, and no mutualists (M–R–) in red. The separation between treatments and clustering within microbial treatments indicate that these mutualists both affect expression system-wide. We note that expression profiles of single-mutualist treatments closely follow their respective regression lines (green, M+R–: $R^2 = 0.98$; blue, M–R+: $R^2 = 0.98$) which pass through the center of the control treatments expression (black ellipse). The 95% confidence interval of the control (M–R–) group means in PC1 and PC2 space is drawn as a black ellipse. (b) Distance from the control group center along the M+R– regression line (M-Score) of each plant in each treatment group. The M–R+ plants did not show movement along the M+R– regression line (i.e. expression unchanged compared to the control), but both the M+R– and M+R+ groups did. Interestingly, the multispecies mutualism shows a significantly greater system-wide shift in gene expression of M-affected genes ($t = -2.514$, $p = 0.026$). Black points and error bars represent group means and standard error of the mean, respectively. (c) Distance from the control group center along the M–R+ regression line (R-Score) of each plant in each treatment group. The M+R– plants did not show movement along the M–R+ regression line (i.e. expression unchanged compared to the control), and while both the M–R+ and M+R+ plants did show directional change in expression along the M–R+ regression line, there was no significant system-wide shifts in expression of R-affected genes ($t = -0.700$, $p = 0.495$).

Figure 12.11.1.3a). The single mutualist-treated plants have high R^2 values (green, M+R–: $R^2 = 0.98$; blue, M–R+: $R^2 = 0.98$) with their regression lines and pass through the M–R– centroid (Figure 12.11.1.3a, black ellipse). These features suggest that movement along these regression lines is primarily driven by independent, mutualist-specific effects; as corroborated by differential expression analysis where ~90% of differentially expressed genes were affected by *either* rhizobia *or* mycorrhizal fungi (see Section 12.11.1.6's Take home 1 below). The slopes of these lines (M+R–: 0.559 ± 0.036; M–R+: -1.307 ± 0.069) are also close to negative reciprocals of each other, indicating that they are approximately perpendicular to one another and thus predominantly describe independent changes in gene expression in response to single mutualists.

After determining that shifts in PC1 and PC2 space of M+R– and M–R+ plants from the control group center represent cumulative shifts of "mutualist independent" gene expression, we calculated the distance of each plant from the control group centroid along each of the separate regression lines (Figure 12.11.1.3b,c). Displacement from the control group center along the mycorrhizal regression (M-Score) and the rhizobial regression (R-Score) line was used as a measure of the cumulative shift from a non-mutualism state to a mycorrhizal- or rhizobial-associating state, respectively (Figure 12.11.1.3b,c). We saw a significant enhancement of mycorrhizal effects in the multiple mutualist-treated plants, i.e. the M-Scores of M+R+ plants are significantly greater than those of the M+R– plants (Figure 12.11.1.3b; $t = -2.514$, $p = 0.026$). This result suggests that co-inoculation of both mutualists enhances the expression of genes that are responsive to mycorrhizal fungi on their own. While a gene-by-gene approach (as described in the following section) is important for identifying strongly responsive candidate genes, subtle changes, like these, may be difficult to detect in a gene-by-gene analysis despite the possibility of a cumulatively important effect on phenotype. However, this was not the case along the R-associated regression, i.e. the R-Scores of M+R+ plants are not significantly different from those of the M–R+ plants (Figure 12.11.1.3c; $t = -0.700$, $p = 0.495$). The synergism created by co-inoculation suggests that multispecies mutualism has many, subtle effects on genes, which at first glance appear to be independently responding to a single mutualist.

12.11.1.6 Independent, additive, and nonadditive effects of multiple mutualists on differential expression of *Medicago truncatula* genes

Multiple mutualists may affect the expression of individual *M. truncatula* genes in at least three ways: independently, additively, and nonadditively (Figure 12.11.1.1c). In the first case, genes are independently regulated by the two mutualists within their own transcriptional networks. In other words, a gene's expression is significantly up or downregulated by a single mutualist. However, the mutualists can also jointly affect gene expression, in additive or nonadditive ways. Additive effects can be modeled by the arithmetic addition of pairwise transcriptional effects. Essentially, both mutualists change expression of the host plant and their effects on expression alone can be summed to determine their effects together.

In contrast, nonadditive effects of multiple mutualists cannot be predicted by summing results from pairwise experiments

or treatments because the effect of one mutualist on expression depends on whether the other mutualist is present. The expression of these genes is significantly greater (or less) than the sum of effects of each mutualist alone, resulting from interactive/indirect effects of multispecies mutualism on gene expression. For example, complementarity between mutualist-conferred benefits (e.g. nitrogen from rhizobia and phosphorus from mycorrhizal fungi) may lead to enhanced plant condition that nonadditively affects host gene expression. Similarly, competition among mutualist partner species, such as mycorrhizal fungi and rhizobia competing for plant-produced photosynthetic carbon, may require transcriptional changes in the plant to regulate these microbial interactions and avoid a transition to exploitation (i.e. inducement of host control mechanisms). Thus, nonadditive effects can result not only from the direct input of multiple mutualist partners, but also by virtue of their interaction.

In our differential expression analysis (Afkhami and Stinchcombe 2016), we examined the prevalence of these three categories and then more deeply explored the genes whose expression was regulated by multiple mutualists. There were five main outcomes from this gene-by-gene analysis:

Take home 1: expression of most genes was regulated by only one mutualist. Mycorrhizal fungi and rhizobia each affected far more genes individually than jointly, with ~90% of differentially expressed genes only being regulated by rhizobia *or* fungi (Figure 12.11.1.4a). Further, mycorrhizal fungi independently regulated the expression of approximately twice as many genes as rhizobia (Figure 12.11.1.4a), although as described in Section 12.11.1.5 (Take home 3), rhizobia may have subtle effects on the expression of some mycorrhizal fungi-responding genes that cannot be detected in this gene-by-gene approach.

Rhizobia-regulated genes were most strongly enriched in gas transport activities (such as oxygen binding and transport which may be important for creating anaerobic nodules; Appleby 1984; Ott et al. 2005) and for protein modifications with kinases and phosphatases. Mycorrhizal-regulated genes showed significant enrichment for metabolic activities and carbon processing, suggesting a link between mycorrhizal fungi and the regulation of plant photosynthesis.

Take home 2: a substantial subset of genes was jointly affected by both symbionts. Approximately 10% (or 623 genes) of the differentially expressed genes were regulated by both mutualists (Figure 12.11.1.4a).

Take home 3: most of the genes regulated by multiple mutualists were affected additively. Of the 623 jointly affected genes, the change in expression for 561 genes was additive (regulatory effect on expression of each symbiont alone could be summed to calculate the joint effect; see Figure 12.11.1.1c, 12.11.1.3a,b). These genes were significantly enriched for "phosphatase activity," which plays an essential role in the uptake of phosphorus by plants (Duff et al. 1994; Ma et al. 2012; Xiao et al. 2006). Changes in the regulation of this enzymatic activity suggest one candidate mechanism for a positive role that this multiple mutualist interaction has on *M. truncatula* performance (Figure 12.11.1.2a,b).

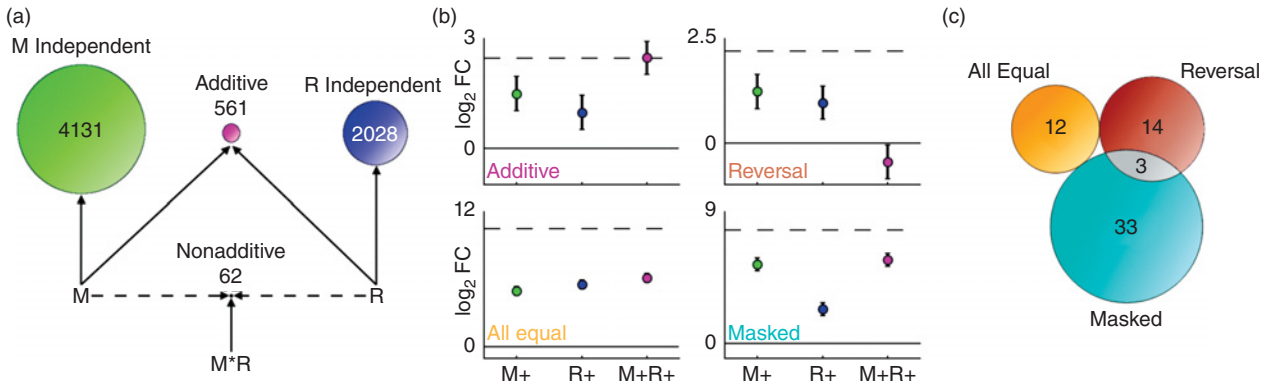

Figure 12.11.1.4 Effects of multiple mutualists on differential expression of genes. (a) Schematic illustrating distribution of mutualist and interaction effects on gene expression. Expression of ~90% of genes was affected by only one mutualist (fungi: green, rhizobia: blue; main effect of rhizobia *or* fungi in differential expression analysis). Another 9% (561; magenta) of genes were additively affected by both mutualists (significant main effects of rhizobia *and* fungi but no significant interactive effect). A small group of genes were nonadditively affected by the multispecies mutualism (significant interactive effect of rhizobia and fungi). Solid arrows indicate significant effect of M (a main effect of mycorrhizal fungi), R (a main effect of rhizobia), or M*R (an interactive effect of mycorrhizal fungi and rhizobia). Dashed arrows used to indicate possible, but not required, significant effects. (b) Examples of multispecies mutualism's effects on expression. Genes were affected additively (*Medtr7g080000*, magenta) or nonadditively. Nonadditive effects can be further divided into reversals (*Medtr3g106060*, brown), equalizations (*Medtr3g078250*, orange), and maskings (*Medtr1g027570*, cyan). See text for descriptions of each category. The y-axes represent $\log_2$(fold change) of treatment compared to expression in control (no mutualist) treatment. On the x-axes, change in expression associated with mycorrhizal fungi (M+) is in green, with rhizobia (R+) is in blue, and with both microbes (M+R+) is in magenta. Circles represent means and error bars represent standard error. Dashed lines represent expected additive effect (i.e. sum of individual fungal and rhizobia effects on expression). (c) Venn diagram displays the breakdown of the nonadditive categories. All but three genes fell into only one category. Most nonadditive genes were represented by masked effects (36, cyan), followed by reversals (17, brown), and equalizations (12, orange), respectively.

Take home 4: multiple mutualists generally "agreed" on the direction of gene regulation. For the genes additively affected by both mutualists, we assessed how often mycorrhizal fungi and rhizobia were likely to be in conflict over the direction of regulation. To do this, we examined the direction of regulation of each gene for plants grown with just rhizobia and for plants grown with just mycorrhizal fungi, finding that for 85% of the genes the microbial mutualists either both upregulated or both downregulated the gene. Potentially conflicting regulatory mechanisms (Figure 12.11.1.1b) were detected in the remaining 85 genes.

Take home 5: multiple mutualists had surprising nonadditive regulatory effects on the expression of a small pool of genes. Ten percent of the jointly affected genes (62 candidate genes) showed effects of the tripartite interaction which did not fit the additive mechanistic model (Figure 12.11.1.4a–c). These nonadditive genes were highly enriched for transport and transporter activities, possibly resulting from reallocation and movement of resources between the host and its symbionts. These nonadditive effects on genes were organized into three categories:

1. **Reversals** consist of directional changes in expression in the tripartite treatment (Figure 12.11.1.3b). Thirty percent (17 genes) of nonadditive genes were reversals (Figure 12.11.1.3b,c) and, in nearly all cases, genes were upregulated in pairwise treatments and downregulated in the multiple mutualist treatment.
2. **All equal** genes showed changes in tripartite treatment expression which were equal to expression changes associated with either mutualist alone (Figure 12.11.1.3c). In other words, mutualists impacted expression but the effect was the same regardless of number of partners and partner identity. All equal genes constituted ~20% (12 genes) of nonadditive genes (Figure 12.11.1.3c).
3. **Masked** genes, in which changes in expression with multiple mutualists was equal to expression with one of the partners (but not both), are the largest category of nonadditive genes (62%; 36 genes) (Figure 12.11.1.3b,c). In the vast majority of cases, the change in expression in response to the multispecies mutualism was equal to change in response to the mycorrhizal fungi (rather than the rhizobia).

Additional details on the gene identities and enrichment analyses for each of the categories described here are available in Afkhami and Stinchcombe (2016).

12.11.1.7 Establishment of multispecies mutualisms and their effects on expression of the shared common symbiosis pathway

Both mycorrhizal fungi and rhizobia utilize a shared common symbiosis pathway in the host plant to establish mycorrhizae and root nodules, respectively (Oldroyd 2013; Zipfel and Oldroyd 2017; see Chapter 8.1). This signaling pathway, which is conserved even in early plant lineages, like Bryophyta and Lycopodiophyta (Wang et al. 2010), is thought to have evolved in response to the more ancient mycorrhizal mutualism and then was later co-opted by rhizobia and other nitrogen fixing bacteria (Oldroyd 2013; Zipfel and Oldroyd, 2017).

To establish these interactions, host plants and symbionts communicate with each other in the rhizosphere (Kosuta et al. 2008; Oláh et al. 2005; Venturi and Keel 2016; Figure 12.11.1.5). Rhizobia and plants respectively release Nod factors and flavonoids to induce establishment of root nodules (Dénarié et al. 1996; Oldroyd and Downie 2008; see Chapters 6.2.1 and 6.2.2) while mycorrhizal fungi initiate colonization by releasing Myc factors after recognizing strigolactones released

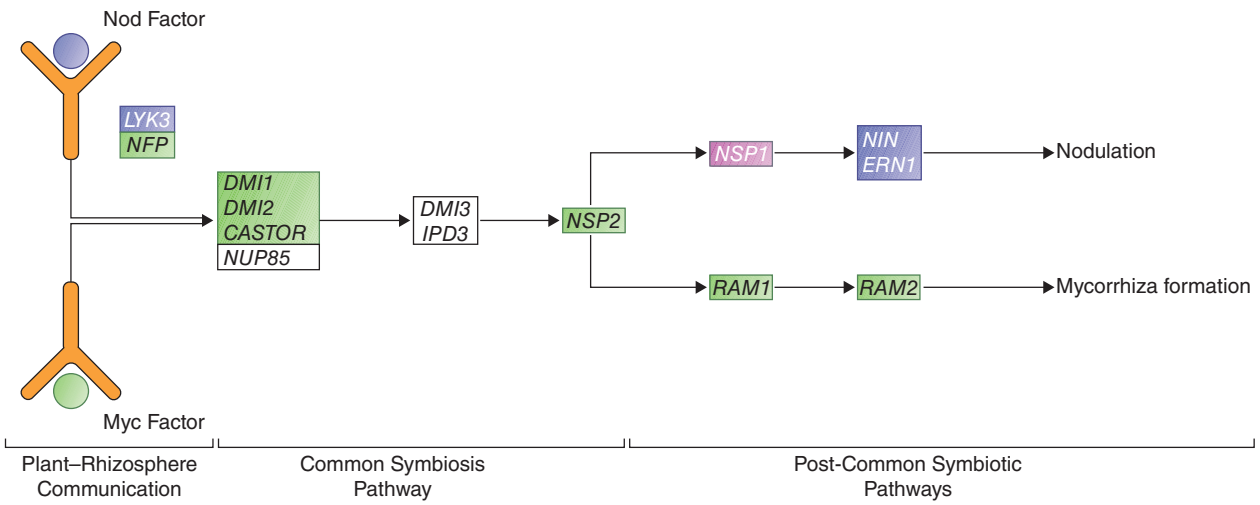

Figure 12.11.1.5 Observed mycorrhizal and rhizobial regulation of common symbiosis pathway. Gene expression of *M. truncatula* common symbiosis pathway was affected by rhizobia (indicated by blue), mycorrhizal fungi (indicated by green), both symbionts (indicated by magenta), or neither (white). Expression of the pathway was more affected by mycorrhizal fungi than rhizobia with fungi impacting expression of half the genes.

by the host plant (Kosuta et al. 2003; Oldroyd et al. 2009; see Chapter 7.1.1).

Following this mutualist-specific rhizosphere communication, both symbionts utilize the host plant's shared common symbiotic pathway which constitutes at least seven genes (*DMI1*, *DMI2*, *DMI3*, *CASTOR*, *NUP85*, *IDP3*, and *NSP2*) important for formation of both symbioses (reviewed in Oldroyd 2013; Figure 12.11.1.5; see also Figure 8.1 and Chapter 8.1). This shared part of the symbiosis pathway is then followed by mutualist-specific pathways to induce nodulation or mycorrhizal formation (rhizobia: *NSP1*, *NIN*, *ERN1*; fungi: *RAM1*, *RAM2*; reviewed in Oldroyd 2013; Figure 12.11.1.5; see Chapters 2.1 and 8.1).

Given what is known about the common symbiosis pathway, we would expect: (i) both mutualists to have significant impacts on expression of genes in shared components of the pathway and (ii) genes in mutualist-specific pathways should only be affected by the relevant mutualist. However, this key pathway could also be an integral regulatory target for interactive effects of host control of multiple mutualists interactions. Here we discuss the three main take homes for the effects of multiple mutualists on regulations of genes involved in plant–rhizosphere communication, the common symbiotic pathway, and post-common symbiotic pathway interactions using the Afkhami and Stinchcombe (2016) data set.

Take home 1: mycorrhizal fungi had a larger impact on expression of this pathway than rhizobia. While neither mutualist altered expression of several shared common symbiosis pathway genes (*NUP85*, *DMI3*, *IPD3*; Figure 12.11.1.5), mycorrhizal fungi changed expression of many other genes involved in establishing symbiosis (8 out of remaining 11). Rhizobia impacted expression of fewer (4 of 11) genes, and rhizobia-associated effects were essentially undetected on shared components of the common symbiosis pathway. All detected regulation of these genes was associated with mycorrhizal fungi which regulated *DMI1*, *DMI2*, *NSP2*, and *CASTOR* (Figure 12.11.1.5). Rhizobia-associated effects were undetected not only in the common symbiosis pathway, but also in some bacteria-specific components during rhizosphere communication (*NFP*). However, it is worth noting that this dichotomy may switch across *M. truncatula* ontogeny with rhizobia-associated signaling and nodulation occurring at earlier time points than was studied in Afkhami and Stinchcombe (2016).

Take home 2: gene expression in the common symbiosis pathway was predominantly influenced by a single mutualist. In fact, only one gene, *NSP1*, was jointly regulated (additively) by rhizobia and mycorrhizal fungi under the conditions of the experiment.

Take home 3: expression of genes in rhizobia-specific components of the nodulation pathway was regulated by the presence of mycorrhizal fungi. In addition to jointly regulating *NSP1*, which is a rhizobia-specific part of the pathway, mycorrhizal fungi altered expression levels of *NFP* which is involved in rhizosphere signaling between plants and rhizobia (Figure 12.11.1.5). The reverse was not observed; rhizobia did not regulate any mycorrhizal fungi-specific pathway components.

We note that all three of our take home conclusions about regulation of the establishment of symbioses and this shared pathway may be strongly affected by timing as use of this pathway by rhizobia and mycorrhizal fungi might vary across host development. For example, mycorrhizal fungi colonization may be important during the time point we examined, nodulation signaling may predominate right after early root elongation, and both may be important, possibly in interactive ways, for a critical time point in the middle. We suggest that examining gene expression of plants factorially inoculated with these symbionts across a time course could provide important insights into ontogenetic changes in signaling through this pathway.

12.11.1.8 Conclusions and future directions

Studies of mutualisms have shown their critical importance in ecological and evolutionary processes (Angelini et al. 2016; Prior et al. 2015) as well as applied value for agriculture and conservation (Derksen-Hooijberg et al. 2017; Hamilton et al. 2016); however, their focus has historically emphasized pairwise interactions. In nature, multispecies mutualistic interactions are pervasive and have the potential to dramatically alter participant fitness and higher-level community and ecosystem processes (van der Heijden et al. 2016). Understanding the mechanistic basis of how plants respond to the extremely common multispecies mutualism with rhizobia and mycorrhizal fungi, using *M. truncatula,* will provide key insights into molecular communication underlying species interactions and the functional roles these interactions play within an ecological and evolutionary context.

Afkhami and Stinchcombe (2016) demonstrated that *M. truncatula*'s growth can respond synergistically to rhizobia and mycorrhizal fungi and that changes in the expression of many genes may underlie the fitness consequences of multispecies mutualisms. Further, this work showed that these multiple mutualists had a wide range of regulatory effects on gene expression, including unexpected reversals and other nonadditive effects. We suggest that several types of studies with *M. truncatula* would improve our understanding of the molecular basis of this complex interaction. First, because molecular pathways are composed of many interacting genes and changes in a single gene's expression may have pleiotropic effects, research into how multispecies mutualisms alter networks of co-expressed genes is important. Second, while whole-organ studies, such as this, provide general insight into averaged/system-wide effects, cellular resolution from functional and transcriptomic studies will be useful in further developing our understanding of multispecies mutualist mechanisms at an organismal level. Cell-specific and multi-tissue transcriptomic approaches would help elucidate differences in how cell types and different tissues

respond to multispecies mutualisms, potentially conveying spatio-temporal information on mutualist effects (both additive and nonadditive) and elucidating multiple mutualist effects in less abundant cell-types obscured in pooled whole-organ studies. For example, multispecies mutualisms may regulate whole-organism synergism by inducing expression changes in specific cell-types, infection stages, or both which can very easily be masked in whole-organ studies with large numbers of unaffected cells/stages. Likewise, investigating expression changes induced by multiple tissues could provide important inferences about the regulation of these interactions. For instance, studies examining expression not only in root, but also leaf tissue could determine the molecular underpinning of resource exchange in this complex interaction, looking at nutrient acquisition functions in roots and photosynthesis-related functions in leaves. Finally, time course studies are critically important for understanding how the interactive effects on expression of this tripartite interaction changes across plant ontogeny. *M. truncatula,* with its symbiotic interactions, tractable biology, and genomic resources, provides an important and representative system for addressing the molecular basis of this ecologically and evolutionarily-important multispecies mutualism.

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