Variation in a microbial mutualist has cascading consequences for host-parasite interactions

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Abstract

Strains of microbial symbionts often vary in their effect on their host. However, little is known about how the genetic variation in microbial symbiont populations impacts host interactions with other co-colonizing microbes. Here we investigated how different strains of nitrogen-fixing rhizobial bacteria affect their host plant's transcriptomic response to parasitic root-knot nematodes in the legume *Medicago truncatula.* Using dual RNAseq of the root organs harboring rhizobia or nematodes, we identified genes from plant host, rhizobia, and nematode whose expression differed between parasite-infected and -uninfected plants, and between plants inoculated with different rhizobial strains.At the site of host-parasite interactions (in nematode galls), hundreds of host genes and few nematode genes differed in expression between host plants inoculated with different rhizobia strains. At the site of host-mutualist interactions (in rhizobia nodules), hundreds of host genes and few rhizobial genes responded to parasite infection. The vast majority of parasite-induced changes in host gene expression depended on the resident rhizobia strain.The transcriptomic changes we observed suggest that microbial indirect genetic effects play an underappreciated role in structuring their host's interactions with other co-colonizing microorganisms, which may be mediated by changes in host resource budgets, defense response, or developmental trajectories.

Keywords: dual RNAseq, host-microbe interactions, indirect genetic effect, intraspecific genetic variation, mutualism, parasitism

Introduction

An individual plant hosts thousands of microorganisms, ranging from bacteria and viruses to eukaryotes like fungi and protozoa. These microbial species are composed of strains that vary – often dramatically – in their effects on their host (Heath, 2010; Heath and Stinchcombe, 2014; Lange et al., 2023; Lively and Dybdahl, 2000; Morand et al., 1997; Osborne et al., 2009; Parker et al., 2017). Here we investigate how strain-level genetic variation in microbial symbiont populations cascades to affect a plant's interactions with other microbes.

A single plant can simultaneously host numerous species of microorganisms. In this way, hosts are ecosystems in which co-colonizing symbionts – parasites, commensals, or mutualists that infect the same host – often compete with or facilitate one another (Coyte et al., 2015; Stein et al., 2013) directly or indirectly via their effects on their shared host (Foster et al., 2017; Pedersen and Fenton, 2007). This framework is implicitly or explicitly employed in a wide range of disciplines studying interactions between hosts and microbial communities: priming of host defenses in plant pathology (Ahmad et al., 2010; Benjamin et al., 2022; Guo and Ge, 2017; Zamioudis and Pieterse, 2012); parasitology, in the context of co-infections or mixed infections (Alizon et al., 2013; Devi et al., 2021; Venter et al., 2022); and community and microbial ecology, as priority effects, indirect effects, or higher-order interactions (Debray et al., 2022; Miller and Travis, 1996; Strauss, 1991). Across these disparate subfields, consensus has emerged from experiments that manipulate the presence of different microbial species within host-associated communities: interactions between co-colonizing microbes of different species have far-reaching ramifications for the assembly and function of host-associated communities (Alizon et al., 2013; Banerjee et al., 2018; Dastogeer et al., 2020; Shetty et al., 2017; terHorst et al., 2018). Co-colonizing microbes of different species influence each other's colonization success (Telfer et al., 2010) and transmission dynamics (Bousema et al., 2008; Pollitt et al., 2015; Schneider et al., 2018), as well as the benefits they confer and costs they inflict on their shared host (Barrett et al., 2015; Larimer et al., 2014; Magnoli and Bever, 2023; Weldon et al., 2020).

However, there is extensive genetic and functional variation within symbiont species. For example, pathogen genotypes differ dramatically in virulence and transmissibility (Lively and Dybdahl, 2000; Morand et al., 1997), strains of bacterial mutualists vary in the costs and benefits they bring to their hosts (Heath, 2010; Heath and Stinchcombe, 2014; Heath and Tiffin, 2007),

and genotypes of defensive mutualists differ in their protective effect (Lange et al., 2023; Osborne et al., 2009; Parker et al., 2017). These examples share a unifying feature: the genes in one species (the microbe) impact trait expression in another (the host). Such "interspecific indirect genetic effects" (De Lisle et al., 2022; Shuster et al., 2006) are thought to be pervasive in traits mediating symbiotic interactions (Fronk and Sachs, 2022) and alter the tempo and mode of trait evolution, because the genetic basis of trait expression and evolution in one species (the host) resides in another species (the microbe) (De Lisle et al., 2022; O'Brien et al., 2021b; Queller, 2014; Shuster et al., 2006).

The extensive genetic variation of symbiosis traits within microbial species raises a crucial question: do different microbial strains of the same species vary in their effect on the host's interaction with other microbes? The answer to this question has broad implications for the assembly, function, and evolution of host-associated communities. If strain-level (i.e., intraspecific) variation in a microbial symbiont influences its host's interaction with other symbionts, then strain-level changes in microbial symbiont populations such as changes in allele frequencies or strain abundance could have cascading impacts on the structure or function of the entire host-associated community. The few studies that have addressed this possibility have found that microbial genotypes do vary in their effect on the broader host-associated community, especially in defensive mutualisms (Lange et al., 2023; Osborne et al., 2009; Parker et al., 2017). However, when two microbes inhabit the same niche within the host (e.g., the animal gut) or colonize systemically, disentangling the host's responses to two co-colonizing symbionts can be prohibitively difficult.

Plant galls provide a unique opportunity to circumvent these challenges and investigate how genetic variation in one microbial symbiont species impacts its host's interactions with other co-colonizing symbionts. Galls are specialized structures that are made of plant tissue but are induced and colonized by another organism such as an insect or microbe. Many plant parasites, pathogens, and mutualists form galls (Harris and Pitzschke, 2020). Common examples include legume root nodules induced by nitrogen-fixing bacteria, goldenrod galls induced by the goldenrod gall fly, and root knot galls induced by root-knot nematodes (Harris and Pitzschke, 2020). These galls are the locus of interaction between the gall inducers and their host plant (Fronk and Sachs, 2022). Because each gall is specific to a single pairwise species interaction,

galls provide a unique opportunity to sample the symbiotic interface between the host and single symbiont.

Transcriptomic methods when applied to galls and nodules are particularly useful in elucidating the otherwise difficult to observe interaction involving hosts and microbes, as they provide genome-wide proxies of the underlying biology mediating the interaction between host and symbiont. In particular, dual RNAseq, where RNA from two symbiotic organisms are sequenced in the same sample, has become a useful tool to investigate in vivo interactions between a host and symbiont (Westermann et al., 2017; Westermann and Vogel, 2021; Wolf et al., 2018). Thus comparing the gene expression profiles of galls can be used to examine changes in the interactions between host plants and their microbial gall-forming symbionts under different conditions.

We leveraged these unique features of plant galls and dual RNAseq to investigate how strain-level variation in a microbial mutualist influences its host plant's transcriptomic response to parasite infection. We inoculated a single genotype of the model legume *Medicago truncatula* with one of two strains of mutualistic nitrogen-fixing rhizobia bacteria *Ensifer meliloti* and infected half of these plants with the parasitic root-knot nematode *Meloidogyne hapla*. We then profiled plant, mutualist, and parasite gene expression in rhizobia nodules and nematode galls (Fig. 1). We asked: (1) how do mutualistic rhizobia influence gene expression at the host-parasite interface in nematode galls?; (2) how do parasitic nematodes affect gene expression at the host-mutualist interface in rhizobial nodules?; and (3) is the effect of parasite infection on nodule gene expression influenced by the resident rhizobia strain?

We found that strain-level variation in the rhizobial mutualist significantly alters the host plant's transcriptomic response to parasite infection. Plants inoculated with different rhizobial strains had differential gene expression in nematode galls, the site of parasite infection. Furthermore, parasite infection altered the expression of hundreds of genes in rhizobia nodules, and this effect depended strongly on the resident rhizobia strain. Our results show that intraspecific genetic variation in a keystone microbial mutualist has cascading transcriptional consequences for host plant interactions with other microorganisms.

Materials and Methods

Study system

Medicago truncatula Gaertn. is an annual legume native to the Mediterranean (Cook, 1999). Upon the transfer of compatible chemical signals, *M. truncatula* plants form associations with nitrogen-fixing rhizobial bacteria in the soil, such as *Ensifer meliloti*. Rhizobia are housed in nodules that develop from the roots of their host plant. Nodules provide the requisite low-oxygen environment and resources for the rhizobia to differentiate into bacteroids within intracellular compartments called symbiosomes. Within symbiosomes, rhizobia fix atmospheric nitrogen into biologically available forms of nitrogen. Hosts provide rhizobia with carbon-containing compounds derived from photosynthate and in exchange receive nitrogen-containing compounds from the rhizobia. The *Medicago-Ensifer* system has become a frequently used and well characterized model of resource-exchange mutualisms (Long, 1989).

The pathogenic root-knot nematode *Meloidogyne hapla* also associates with *M. truncatula* roots. Female *M. hapla* burrow through the intercellular spaces of host roots in search of a suitable infection site. There *M. hapla* stops and uses its stylet mouthpiece to inject effector proteins into parenchymal host cells and root tissues that suppress immune responses and induce cell differentiation into giant cells from which the pathogenic nematode siphons nutrients. Galls form around the site of infection (Jones and Goto, 2011).

We chose the in-bred line of *M. truncatula* HM145 from the French National Institute for Agriculture Research germplasm library for its high nodulation rate and nematode susceptibility (Wood et al., 2018). We obtained inoculum containing the northern root-knot nematode *M. hapla* line LM originally collected in France and supplied by the Department of Entomology and Plant Pathology at North Carolina State University. We used *E. meliloti* strains USDA1021 and WSM1022 from lab stocks (JRS). These two strains show a genomic 31-kmer Jaccard distance of 0.523 or 47.3%, an average nucleotide identity similarity of 98.27%, and the genomes show a Jaccard distance in gene presence or absence of 0.718 with 7,653 genes in the pangenome and 5,496 orthologous genes. See Supporting Information for details about how these similarity scores were calculated (Methods S2).

Sample preparation

Per Garcia *et al*. (2006), we mechanically scarified *M. truncatula* HM145 seeds with sandpaper, sterilized them with 10% bleach solution, stratified them in the dark at 4 °C for 2 days on sterile water agar plates, and then incubated them at room temperature for 2 days. We planted germinated seedlings in 120 mL Cone-tainers that had been filled with 1:4 mixture of perlite and sand and autoclaved twice with >24 hours between each autoclaving (Barker et al., 2006). We maintained seedlings in growth chambers at the University of Pennsylvania in a randomized complete block design of rhizobia strain (USDA1021 or WSM1022) and nematode status (N+ or N-). Each of the four treatment types (USDA1021 N+, WSM1022 N+, USDA1021 N-, and WSM1022 N-) had three replicates with each replicate consisting of organ samples pooled from six individual plants. Growth chambers were kept in a 16:8 light:dark cycle, alternating between 25 °C during the light cycle and 21 °C during the dark cycle. After planting we fertilized plants on a recurring basis with details of the fertilizing procedure and schedule found in Supporting Information (Table S2, Methods S3).

On day 14 after planting, we inoculated each *M. truncatula* plant with its corresponding rhizobia and nematode treatments. Nematode inoculation consisted of 5 mL of double deionized water solution containing 86 nematode eggs mL^{-1} (~430 eggs total). Rhizobial inoculation consisted of 5 mL of 2 day old liquid culture in tryptone yeast media, diluted to OD600 of 0.1 (Journet et al., 2006). Plants not inoculated with nematodes were given 5 mL of double deionized water. Plants were fertilized on subsequent days with details of the fertilizing procedure and schedule found in Supporting Information (Table S2, Methods S3).

Organ sample harvesting

Organ samples were collected from every surviving plant 52-53 days after planting. All six plants that constituted a given replicate were harvested on the same day. Nodule samples were collected from all plants and root and gall samples were collected from plants inoculated with both rhizobia and nematodes (Fig. 1). To harvest, the root system was washed in water, then organs were manually excised using a pair of sterilized scissors and immediately flash frozen in liquid nitrogen. Samples were taken from the top ⅓ of the root system so as to collect galls and nodules at a consistent level of maturity. All samples were stored at -80 °C until RNA extraction which was performed within 30 days.

Dual RNA sequencing of nodules and galls

To isolate species interactions in co-colonized hosts, we extracted RNA from nodules and galls and performed dual RNAseq with nematode, plant and bacterial rRNA depletion on pooled organ samples. We used rRNA depletion as opposed to polyA-selection so as to preserve non-polyadenylated prokaryotic reads, enabling quantification of gene expression in both the eukaryotic plant host and eukaryotic nematode parasite in galls, and both the eukaryotic plant host and prokaryotic bacterial mutualist in nodules. To assess the localized or systemic nature of host changes in gene expression, we also performed RNAseq with rRNA pull-down on samples of roots from co-colonized hosts.

We used a Trizol and QIAGEN RNeasy Kit based RNA extract. See Supporting Information for details on RNA extraction (Methods S4). We assessed the quality of all RNA extracts using a Nanodrop ND-1000 (Thermo Fisher Scientific) and found that all extracts were of sufficient quality for sequencing (absorbance at 260 nm / 280 nm between 1.8 and 2.2). RNA extracts were shipped to Genewiz Azenta Life Sciences (South Plainfield, NJ) for sequencing. We distributed RNA samples from the same treatment type across different lanes to preclude sequencing lanes as a batch effect. Raw read data was uploaded to the Sequence Read Archive under BioProjectID PRJNA1090526.

Dataset filtering and gene expression quantification

After sequencing, we used the default settings of Trimmomatic (Bolger et al., 2014) to remove sequencing adapters from reads and discard poor quality reads. We used Sortmerna (Kopylova et al., 2012) and the rRNA sequences of all three species in the study system in the SILVA database (Glöckner et al., 2017; Pruesse et al., 2007; Quast et al., 2013; Yilmaz et al., 2014, p. 2) to remove rRNA reads that were not removed by rRNA depletion. The remaining reads were aligned with default settings to eukaryotic genomes using HISAT2 (Kim et al., 2019) and to prokaryotic genomes using bowtie2 (Langmead and Salzberg, 2012). Read alignments with quality score <30 and whose paired mates aligned to separate chromosomes were discarded. The remaining alignments were used with the union method from HTSeq-count (Putri et al., 2022) to quantify gene expression of all genomic features not annotated as repeat regions, rRNA, or tRNA in reference genomes. See Supporting Information for reference genome information and greater details about software specifications (Methods S2).

After gene expression quantification, we found all quality metrics and statistics to be suitable for downstream analyses (Chung et al., 2021; Love et al., 2014): host gene expression quantification data segregated on a principal component analysis by sample type as expected (Fig. S1) and the percentage of host genes with a coefficient of variation across individual treatments that is greater than across all samples never exceeded 20% for a single sample (Table S5), indicating that biological variation was greater than technical variation for the large majority of genes.

Differential expression analyses

One convention for categorizing eukaryotic gene expression is to consider genes with an aligned read count of >1 RPKM (aligned reads per kilobase of gene length per million reads) as expressed (Hebenstreit et al., 2011). For consistency, we applied this categorization to all plant, rhizobia, and nematode genes (with total millions of reads used to calculate RPKM being equal to the millions of reads aligning to a given organism's genome). To identify differentially expressed genes (DEGs), we used DESeq2 (Love et al., 2014) with a likelihood ratio test on all genes expressed in a given organ. We used several DESeq2 models to test for differential expression across different sample types (Methods S1, Table S2 and S4)

Gene ontology overrepresentation analyses

We used Gene Ontology overrepresentation analyses (GOORA) of gene lists generated from our differential expression analyses to formulate hypotheses about the biological processes that may be different across our treatment types. For each *M. truncatula* organ in our analysis, we created a custom database of the genes that were expressed in that organ and their corresponding Gene Ontology annotations from the *M. truncatula* genome annotation. We used the default settings (p value 0.05, q value 0.2, Benjamini–Hochberg correction) of the enrichGO function from the clusterProfile package in R (Wu et al., 2021) against the aforementioned databases to identify over represented Gene Ontology terms in the gene lists generated by our differential expression analyses.

Results

Mutualist strain impacts the expression of host genes related to defense, development, and pathogenesis at the site of host-parasite interactions.

Plant host gene expression in nematode galls was different in hosts inoculated with different rhizobia strains. Out of the 51,653 examined genes in the *M. truncatula* genome, 25,614 genes (49.589%) were expressed. Of these genes, 261 genes (1.02%) were differentially expressed (DE). Of these DEGs, the majority (179 genes; 68.6%) had higher expression in hosts inoculated with WSM1022 than hosts inoculated with USDA1021 (Fig. 2a, c). Two of the DEGs were only expressed in hosts with USDA1021 (a guanine deaminase MtrunA17_Chr1g0190991, and a phosphate transporter MtrunA17_Chr1g0167761).

GOORA showed annotations associated with diverse processes are overrepresented in all 261 host DEGs that responded to rhizobial strain differences in nematode galls (Dataset S**6**). Notably, functions related to the plant hormone abscisic acid, which coordinates plant responses to biotic and abiotic stress (Cao et al., 2011), were overrepresented. Functions related to metabolism of cell wall components such as xylan, hemicellulose, arabinan, and pectin, and cell wall organization and biogenesis were also overrepresented. Changes in plant cell walls are both a symptom of and defense response against root knot nematode infection (Caillaud et al., 2008; Przybylska & Obrępalska-Stęplowska, 2020). Phosphoprotein regulation was also overrepresented in the host gene expression changes (Table S1a; see Dataset S6 for full details of GOORA results).

GOORA produced similar results when performed with only the DEGs in nematode galls that were upregulated in hosts inoculated with USDA1021. For the DEGs in nematode galls that were upregulated in hosts inoculated with WSM1022 only carbohydrate metabolism (GO:0005975) and galacturan 1,4-alpha-galacturonidase activity (GO:0047911) were overrepresented (Dataset S**6**). The activity of galacturan 1,4-alpha-galacturonidase degrades pectin in plant cell walls (Hasegawa and Nagel, 1968). Galacturan 1,4-alpha-galacturonidase enzymes are also secreted by root knot nematodes into cells at the infection site and pectin de-esterification and degradation is critical for successful root knot nematode infection (Jagdale et al., 2021; Wieczorek et al., 2014). Pectin metabolism and homeostasis affects many developmental pathways in plants (Guo et al., 2022; Wolf et al., 2009). Together these ORA

results suggest the possibility that host developmental processes, defense responses, or host pathogenesis are altered by different rhizobia strains.

Mutualist strain has little impact on global parasite gene expression but does alter a putative parasitism gene

Nematode gene expression showed little response to differences in the rhizobia strains resident in host nodules. Out of the 14,419 genes in the *M. hapla* genome, 14,230 genes (98.689%) were expressed under the aforementioned expression criteria. Of these genes, only six (0.04%) were DEGs (Fig. 2b, Table 1a).

Many parasitic nematode effector proteins localize to the nucleus where they modulate host gene expression, so we investigated the predicted protein products of the six differentially expressed genes for secretory signal sequences using SignalP 6.0 (Teufel et al., 2022) and for nuclear localization signals using NLStradamus (Nguyen Ba et al., 2009). The β-lactamase HcpB-like gene (MhA1_Contig1719.frz3.gene12) and predicted rhodopsin-like G-protein coupled receptor gene (MhA1_Contig543.frz3.gene1) have nuclear localization signal sequences but are not predicted to be secreted proteins. The protein product of the predicted cylicin-2 gene (MhA1_Contig253.frz3.gene16) is predicted to be a secreted protein. This gene shows significant sequence similarity (coverage of 95% and percent identity 81.13%) to Minc00331 in the closely related *Meloidogyne incognita* which is presumed to be a parasitism gene as it is upregulated during parasitic life stages (Nguyen et al., 2018).

Mutualist strain variation elicits similar responses in nodules and galls

Out of the 20,116 *M. truncatula* genes expressed in all three organ types, 18 genes showed a similar response to rhizobia strain across all organ types (Table 1c) and 861 genes were DEGs in response to rhizobia strain in at least one organ type (a main effect of rhizobia strain or an rhizobia strain by organ interaction effect respectively). This indicates that the response to different mutualist strains varies across organs and that very few host genes are responding to intraspecific genetic variation in a symbiotic microbial mutualist similarly in all organs.

Few genes are responding to differences in mutualist genotypes in similar ways across roots, nodules, and nematode galls, but similar responses can be observed across pairs of organs. Out of the 28,292 *M. truncatula* genes expressed in both symbiotic structures – nodules and galls – 712 genes showed a similar response and 136 genes showed a different response to mutualist

strain (Fig. 2d), indicating that the majority of genes that respond to different mutualist strains in galls and nodules (84.0%) are responding in similar ways. Despite the lack of systemic response to different rhizobia strains across all organs in this study, symbiotic organs are responding similarly to differences in rhizobial strain. GOORA of the genes responding similarly in galls and nodules suggests that both symbiotic organs have similar changes in their sugar metabolism, cell walls, DNA transcription, and protein phosphorylation (Dataset S6). When changes in galls were compared to changes in roots, we found that out of the 27,298 *M. truncatula* genes expressed in galls and roots, 192 genes showed a similar response and 34 genes showed a different response to rhizobial strain across the two organ types (Fig. 2e), indicating that the majority of genes in galls and roots that respond to rhizobial differences (85.0%) are responding to mutualist strain in similar ways in the two organs.

Parasite infection impacts gene expression at the site of host-mutualist interactions in both strains of rhizobia

In nodules with rhizobia strain USDA1021, 23,848 *M. truncatula* genes (46.170% of the genome) were expressed. Of the expressed genes, 1,428 host genes (5.988%) were differentially expressed and similar numbers were up- and down-regulated in response to parasite infection (773 and 665 genes respectively) (Fig. 3a). Of these responding genes, 122 of them (8.45%) were conditionally expressed with 42 expressed only when parasites are present and 80 expressed only when parasites were absent.

GOORA suggests that annotations associated with diverse processes are overrepresented in all 1,428 responsive host genes, but notably, a number of important biological functions integral to legume-rhizobia mutualism were overrepresented. These annotations include Gene Ontology terms for the metabolism of various types of sugar (including disaccharide, oligosaccharide, and glycogen metabolisms), energy reserve metabolism, abscisic acid response, defense response, transmembrane transport, structural constituents of cell walls, iron ion binding, and tripeptidyl-peptidase activity and the extracellular region, cell periphery and apoplast as cellular context. When subsetting this gene list by upregulation and downregulation, there were similar overrepresentation results. However, genes that were upregulated in infected plants were overrepresented with annotations for water transport related terms, amino acid transmembrane transport, and sucrose synthase, and genes that were downregulated in infected plants overrepresented with annotations related to response to water deprivation, metabolism related to

sterols, oligopeptide transport, apocarotenoid metabolism, and calcium activated cation channel activity (Tables **1b**, **c**, **d**; see Dataset S**6** for full details of GOORA results).

In nodules with WSM1022, 23,673 *M. truncatula* genes (45.831% of the genome) were expressed. Of the expressed genes, 386 genes (1.63%) were differentially expressed and the majority (358 genes; 92.8%) were upregulated in response to parasite infection (Fig. 3a). Of the responding genes, 20 of them (5.18%) were conditionally expressed with 3 of them expressed only in the presence of parasites (MtrunA17_Chr3g0097031, MtrunA17_Chr3g0134401, MtrunA17_Chr5g0422111) and 17 of them expressed only in the absence of parasites. Of these conditionally expressed genes, two of them (a cyclin A gene, MtrunA17_Chr3g0134401, and putative protein, MtrunA17_Chr5g0422111) were also conditionally expressed in nodules with USDA1021 and expressed only in the presence of parasites.

Our GOORA suggests that parasite infection may impact the expression of host defenses as annotations related to defense were overrepresented in all 386 responsive host genes (Dataset S**6**). Terms related to defense response, salicylic acid metabolism and its regulation, response to chitin, stress response, signal transduction, transcription related, and cell wall related terms were overrepresented. GOORA of host genes that were downregulated in response to parasite infection showed similar overrepresentation results whereas upregulated host genes showed very few overrepresented Gene Ontology terms (Table S1e; see Dataset S6 for full details of GOORA results).

In contrast to the host, parasite inoculation caused little change in gene expression in rhizobia. WSM1022 showed no DEGs and USDA1021 had only 13 DEGs (Fig. 3b, Table 1b).

Parasite infection impacts host gene expression at the site of host-mutualist interactions in a largely strain specific manner

A parasite's strain-specific impact on host gene expression in nodules has the potential to influence host-mutualist interactions. Out of the 24,579 host genes expressed across all nodule samples, 193 genes (0.785%) responded similarly to parasite infection in nodules regardless of mutualist genotype. On the other hand, 1,632 of the expressed genes (6.640%) showed a strain specific response to parasite infection. This indicates that the majority of genes responding to parasite infection in nodules (89.42%) respond in different ways to mutualist genotype (Fig. 3d). Notably many of these genes responded in opposite ways when different rhizobia were resident

in the nodule. Of these genes showing a strain-specific response, 90 genes (5.5%) showed conditional expression in which the gene was not expressed in at least one treatment type.

GOORA showed that annotations potentially involved in host-mutualist interactions are overrepresented in the host genes showing a strain specific response to parasite infection. Notably, terms related to stress and defense responses, response to other organisms, cell communication and signal transduction, protein phosphorylation, autophosphorylation, and kinase activity, carbohydrate and energy reserve metabolism of various types as well as the transferase or hydrolyzing activity of glycosyls or sugars, and various processes related to components or organization of the cell wall were overrepresented. Additionally, Gene Ontology terms related to cellular context showed that the genes showing a strain specific response were overrepresented for anchored components of the plasma membrane, plasmodesma, and cell walls (Table S1f; see Dataset S6 for full details of GOORA results). As many of these functions are important for plant-rhizobia interactions, especially carbohydrate metabolism (Udvardi and Poole, 2013) and defense responses (Cao et al., 2017; Gourion et al., 2015), this suggests that the strain-specific effect of parasites on host gene expression in nodules may influence host-mutualist interactions. Notably, the leghemoglobin gene MtrunA17_Chr5g0427351, a critical component of creating an environment within nodules that is conducive to nitrogen fixation (Larrainzar et al., 2020), responded to parasite infection in a strain-specific manner (LFC of 0.7 in nodules with USDA1021 and LFC of -1.2 in nodules with WSM1022), further supporting the above hypothesis.

Nodule cysteine-rich protein gene expression is impacted by parasite infection

Nodule cysteine-rich (NCR) proteins are an important group of defensin-like proteins that are unique to legumes (Downie and Kondorosi, 2021). NCR proteins are highly expressed in and only in nodules (Guefrachi et al., 2014), and often play critical roles in coordinating the legume-rhizobia mutualism and nodule development and homeostasis (Burghardt, 2020; Burghardt et al., 2018; Durgo et al., 2015; Pan and Wang, 2017; Roy et al., 2020). Out of the 840 annotated NCR protein genes in the genome used in this analysis, 392 NCR protein genes (46.7%) were expressed across all nodule samples, and 74 (8.81%) were expressed across at least one but not all nodule samples. Of all 466 expressed NCR protein genes, 83 (17.8%) were differentially expressed in nodules in at least one of the models used in this study. Of these 83 differentially expressed NCR genes, 54 responded to nematode infection status in at least one

model, 63 responded to rhizobial strain differences in at least one model, 34 responded to both nematode infection status and rhizobial strain differences in at least one model, and 29 responded in a strain-specific manner (i.e., an infection × rhizobia strain interaction). Three of the differentially expressed NCR protein genes (MtrunA17_Chr3g0083081, MtrunA17_Chr3g0083091, and MtrunA17_Chr3g0083111) are present in a region of chromosome 3 that causes defective nitrogen fixation when deleted (Shen et al., 2023) and all three had a response to rhizobial strain differences or nematode infection in at least one model.

Discussion

Here, we showed that intraspecific genetic variation in a major microbial mutualist impacts the host plant's transcriptomic response to parasite infection. At the site of parasite infection (nematode galls), hundreds of genes were differentially expressed across plant hosts that were inoculated with one of two different strains of mutualistic rhizobia. At the site of mutualist colonization (rhizobial nodules), the effect of parasite infection on host gene expression depended on the resident mutualist strain. Our results suggest that microbial indirect genetic effects play an underappreciated role in structuring their host's interactions with other co-colonizing microorganisms.

Microbial mutualists shape their host's transcriptomic response to parasite infection

Strain-level variation in a microbial mutualist was a major determinant of gene expression in parasite-infected hosts. This effect manifested in two ways. First, rhizobia had a comparable effect on host gene expression in nematode galls as in rhizobia nodules. The number of rhizobia-responsive plant genes in galls was greater than in nodules, despite the magnitude of differential expression being slightly larger in nodules (Fig. 2c). Second, rhizobia largely determined the effect that parasite infection had on host gene expression. Hundreds of genes that were upregulated in response to parasite infection when the host was inoculated with one rhizobial strain were downregulated in response to parasite infection when inoculated with another strain (Fig. 3c).

Our results show that microbial effects on host gene expression extend to other species interactions their host engaged in. Furthermore, microbial effects on a host's third-party interactions can rival their direct effect on hosts at their site of colonization. These results are consistent with and build on the growing body of evidence that microorganisms can strongly

influence gene expression in their hosts: microbial modulation of host gene expression has been demonstrated in terrestrial and marine mutualisms (Burghardt et al., 2017; Heath et al., 2012; Palakurty et al., 2018; Rodriguez-Lanetty et al., 2006), in animal and plant pathogens (Apidianakis et al., 2005; Stringlis et al., 2018), and in the primate microbiome (Grieneisen et al., 2020).

The consequences of cascading effects of mutualist strain variation

Simple models of selection acting on mutualisms can erroneously produce expectations that selection should remove intraspecific variation in mutualism-related traits. Yet, in reality, extensive intraspecific variation is a hallmark of many mutualism-related traits (Heath and Stinchcombe, 2014). This is true in the legume-rhizobia mutualism as rhizobia strains vary dramatically in the fitness benefit they provide their host (Heath, 2010). Similar patterns have been documented in mutualisms across the tree of life, from mycorrhizal fungi (Hart et al., 2013), to obligate pollinators (Jandér et al., 2012), to the bacterial symbionts of marine mussels (Ansorge et al., 2019). This disparity has motivated searches for mechanisms that maintain variation in mutualisms (Heath and Stinchcombe, 2014). Our experiment suggests that these mechanisms maintaining variation in mutualisms may have unanticipated and underappreciated consequences for the ecological communities in which mutualisms are embedded. Ultimately, however, predicting when and how intraspecific variation in mutualisms will cascade to impact other species interactions in host-associated communities requires understanding the mechanisms mediating crosstalk between microbial mutualists and other co-colonizing microorganisms.

Potential mechanisms underlying mutualist strain effects on host-parasite interactions

Why might strain-level variation in a microbial mutualist have such a large effect on the host's response to a parasite? We elaborate on three non-exclusive explanations below: a defense-centric, resource-centric, and development-centric model.

First, mutualist strains may elicit different defense responses in their host that may cascade to impact the expression of defense genes at the site of parasite infection. The establishment and maintenance of many mutualisms requires tightly coordinated control of the host's immune response (Zipfel and Oldroyd, 2017). In the legume-rhizobia mutualism, suppression of the plant's immune response via nod factors and other effectors orchestrate the initiation and progression of the mutualism (Cao et al., 2017; Gourion et al., 2015). Mature

nodules have a suppressed immune response, and functional genetic experiments have established that maintaining a functional symbiosis necessitates a controlled and tightly coordinated immune response (Zhang et al., 2023).

Consistent with this defense-centric model, our GOORA analysis of gall samples uncovered enrichment of defense-related functions in DEGs. Notably, functions related to the plant hormone abscisic acid were overrepresented. Abscisic acid coordinates biotic and abiotic stress responses (Cao et al., 2011), although its role in plant responses to nematode infection remains poorly understood (Gheysen and Mitchum, 2019). changes in plant cell walls are a defense response against root knot nematode infection (Caillaud et al., 2008; Przybylska & Obrępalska-Stęplowska, 2020) and functions related to the metabolism of cell wall constituents were also overrepresented.

Second, mutualist strains may differ in the resources they provide or costs they impose on their host. If strains differ in their impact on the host's resource budget, these differences could cascade to impact resource flux at the site of parasite infection or produce differences in the way mutualism resource fluxes respond to parasite infection. Intraspecific variation in the costs and benefits a mutualist provides is well documented in the legume-rhizobia mutualism (Heath, 2010). Rhizobia strains vary in the nitrogen and benefits they provide their plant host (Batstone et al., 2017; Terpolilli et al., 2008). If the strains in our experiment negotiated different carbon-for-nitrogen exchange rates with their host, the resulting impact on the host's resource budget could have driven differences in resource flux in nodules, galls, or both, driving the transcriptomic differences we observed.

In support of this resource-centric model, our GOORA analyses suggest that rhizobia strain affected carbohydrate metabolism in galls and nematode infection affected carbohydrate metabolism in nodules (Dataset S**6**). Carbohydrate metabolism plays a central function in the legume-rhizobia mutualism (Udvardi and Poole, 2013), and root knot nematodes siphon carbohydrates from the phloem, facilitated by active sugar transport into galls (Zhou et al., 2023). Furthermore, the expression of leghemoglobin, a key regulator of the nitrogen-fixation reaction in rhizobia nodules (Larrainzar et al., 2020), exhibited a strain-specific response to parasite infection. This observation raises the intriguing hypothesis that rhizobia strains control the sensitivity of nitrogen fixation—the central function of the legume-rhizobia mutualism—to parasite infection.

Third, mutualist strains may differ in their impact on developmental processes. Symbiotic microbes are known to have profound effects on host development in both plants and animals on a systemic and local level (Fronk and Sachs, 2022; O'Brien et al., 2021a; Sommer and Bäckhed, 2013). Furthermore, resistance to infectious disease changes across ontogeny: juveniles are typically more susceptible to infection than adults (Ashby and Bruns, 2018; Izhar et al., 2020). If mutualist strains have different impacts on host systemic ontogeny and host ontogeny influences parasite resistance or infection trajectory, then hosts inoculated with different mutualists could be in different stages of infection when sampled at the same chronological age. On a local scale, mutualist strain differences may alter gall or nodule development. Regardless of scale, in bulk RNAseq, differences in ontogeny can produce signals of differential expression if the ratio of different cell types in sequenced bulk organs differs across treatments of interest (Hunnicutt et al., 2022; Montgomery and Mank, 2016). Therefore, the differential expression that we observed may result from rhizobia strains influencing gall, nodule, or systemic development such that cell composition in galls or nodules differed across treatments.

Outstanding questions and future directions

Our results suggest several promising avenues of future research. First, what are the relative contributions of defense-, resource-, and development-centric models to crosstalk in host-associated communities? Our GOORA implicates both the defense- and resource-centric models in mediating transcriptional crosstalk between the rhizobia mutualism and nematode parasitism but is unable to implicate development-centric models. Future experiments should independently manipulate host defense and resource flux to disentangle their independent contributions to crosstalk between co-infecting mutualists and parasites. Sequencing technologies that de-confound the gene expression profiles of different cells or tissues, such as spatial transcriptomics (Rao et al., 2021) or single-cell RNA-seq (Saliba et al., 2014), could also shed light on the contribution of development to differential gene expression. Beyond illuminating the organismal processes that underlie crosstalk in host-associated communities, discriminating between defense-, resource-, and development-centric models is important to understand how these communities may evolve, as different underlying mechanisms implicate different regimes of pleiotropy and crosstalk that may constrain or alter evolutionary potential is able to shape organismal traits and species interactions. For example, if crosstalk is primarily mediated by the host's defense response, co-colonization may impact the evolution of genes and

processes involved in host immunity. Alternatively, if crosstalk is primarily resource mediated, co-colonizers may influence the evolution of resource allocation and acquisition traits in their hosts.

Second, why was the transcriptomic response to co-colonization attenuated in symbionts relative to hosts? And why were changes in host gene expression in symbiotic structures not mirrored by the resident symbiont? In contrast to host gene expression, mutualist gene expression was largely unaffected by parasite infection, and parasite gene expression was largely unaffected by mutualist strain differences. Only 0-0.2% of rhizobia and nematode genes responded to their co-colonizer, while 1-6% of plant genes responded to variation in one or both symbionts. This observation suggests that the effect of mutualist strain on host-parasite interactions is primarily driven by the host. One straightforward explanation is that direct contact between species drives a larger transcriptomic response than indirect interactions. Another possibility is that generalist symbionts — like root-knot nematodes (Castagnone-Sereno et al., 2013; Catella et al., 2022), and, to a lesser extent, *Ensifer* rhizobia (Harrison et al., 2018) — have evolved transcriptional robustness across a wide variety of host environments.

Third, when does crosstalk in host-associated communities arise from systemic versus symbiosis-specific host responses? A significant insight from our experiment is that mutualist effects on gene expression at the site of parasite infection were specific to symbiotic organs and not a byproduct of systemic changes in host gene expression. Rhizobia elicited a different transcriptomic response in nodules and galls than in uncolonized root tissue (Fig. S2). One possible explanation is that symbiosis-specific responses dominate whenever there is substantial overlap in the function of mature symbiotic structures induced by different colonizing species. Despite rhizobia being a mutualistic prokaryote and nematodes being a parasitic eukaryote, there is substantial overlap in their functions (Fronk and Sachs, 2022): both involve nutrient exchange, manipulation of the host defense response, and modification of host cell development and differentiation (Koltai et al., 2001). Regardless of the mechanism, our results underscore the importance of tissue specificity when studying the transcriptomic basis of host-symbiont interactions (Koltai et al., 2001; Przybylska and Obrępalska-Stęplowska, 2020).

Finally, our study raises a final fundamental question: How much genetic variation for traits mediating species interactions (e.g., parasite resistance and mutualism function) resides in species not directly involved in the interaction itself? Our results indicate that indirect genetic

effects are important and overlooked coordinators of species interactions within multispecies contexts. The concept of indirect genetic effects—when one organism's genes influence another's phenotype—was originally formalized in the context of intraspecific interactions like aggression or maternal effects (Moore et al., 1997; Wolf et al., 1998). Recently, this framework was formally extended to interactions between species (De Lisle et al., 2022). However, this field primarily remains focused on pairwise species interactions: for example, how host genes influence parasite virulence or the benefit derived from a mutualistic symbiont. A significant insight from our study is that indirect genetic effects in species interactions extend to third parties. Given the potential that microbial communities have for impacting selection and overall health of their hosts (Lau and Lennon, 2011), future work should explore the role of intraspecific variation in microbial symbionts in shaping the structure and function of host-associated communities and its consequences for host health and fitness.

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Data Accessibility and Benefit-Sharing

Data Accessibility Statement

Raw sequence reads are available through the Sequence Read Archive under BioProjectID PRJNA1090526. Scripts for data analysis and the generation of figures and datasets are available at www.github.com/adbmar/MtruncDualRNASeq2024.

Benefit-Sharing Statement

Benefits from this research accrue from the sharing of our results and the data and analytical pipelines found on public databases as described above.

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Author Contributions

EGT, CWW, and JRS contributed to the conception of the project; EGT and CWW designed the experiments; EGT and EY carried out the experiments; EGT and ADBM extracted RNA; ADBM performed data analysis; ADBM and EY crafted figures; ADBM wrote the first draft of the manuscript and all authors contributed to revisions.

Tables and Figures

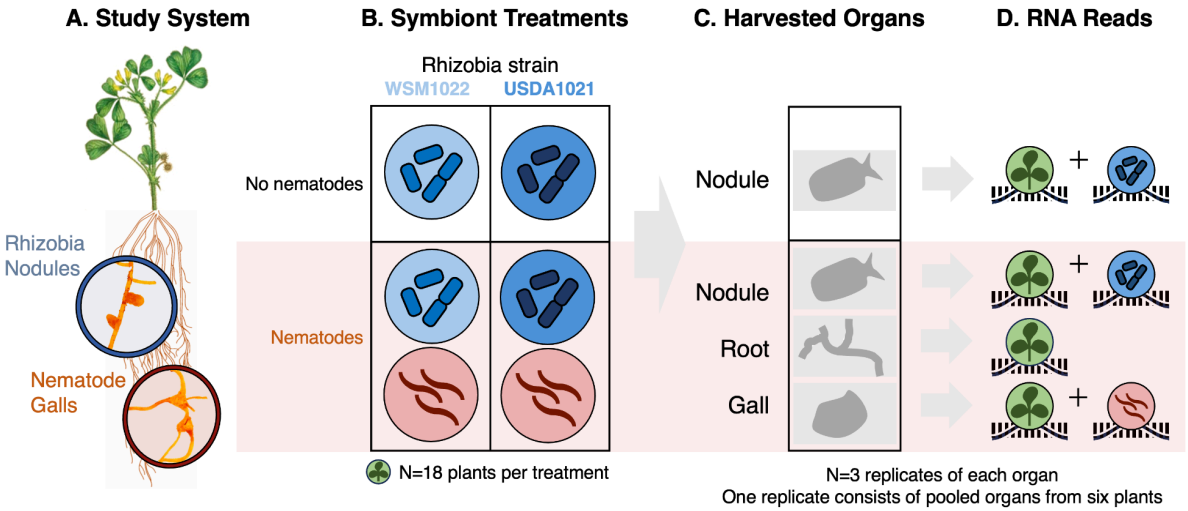

Figure 1: Conceptual diagram outlining the experimental design, treatment types, and samples used in this experiment. A) The host plant, *Medicago truncatula*, with rhizobia nodules and nematode galls in the host plant root system as magnified insets. B) The four treatment types of symbionts used in this experiment: USDA1021 N-, USDA1021 N+, WSM1022 N-, WSM1022 N+. Each treatment type consisted of 3 biological replicates with six plants constituting each biological replicate, totaling 18 plants per treatment type. C) The organs harvested from the treatment types: rhizobia nodule and root samples were harvested from hosts that were not inoculated with nematode parasites and rhizobia nodule, root, and nematode gall samples were harvested from hosts that were inoculated with nematode parasites. D) The organisms of origin of the recoverable RNA reads from each organ. Prior to RNA extraction, organs from the six plants constituting one biological replicate were pooled.

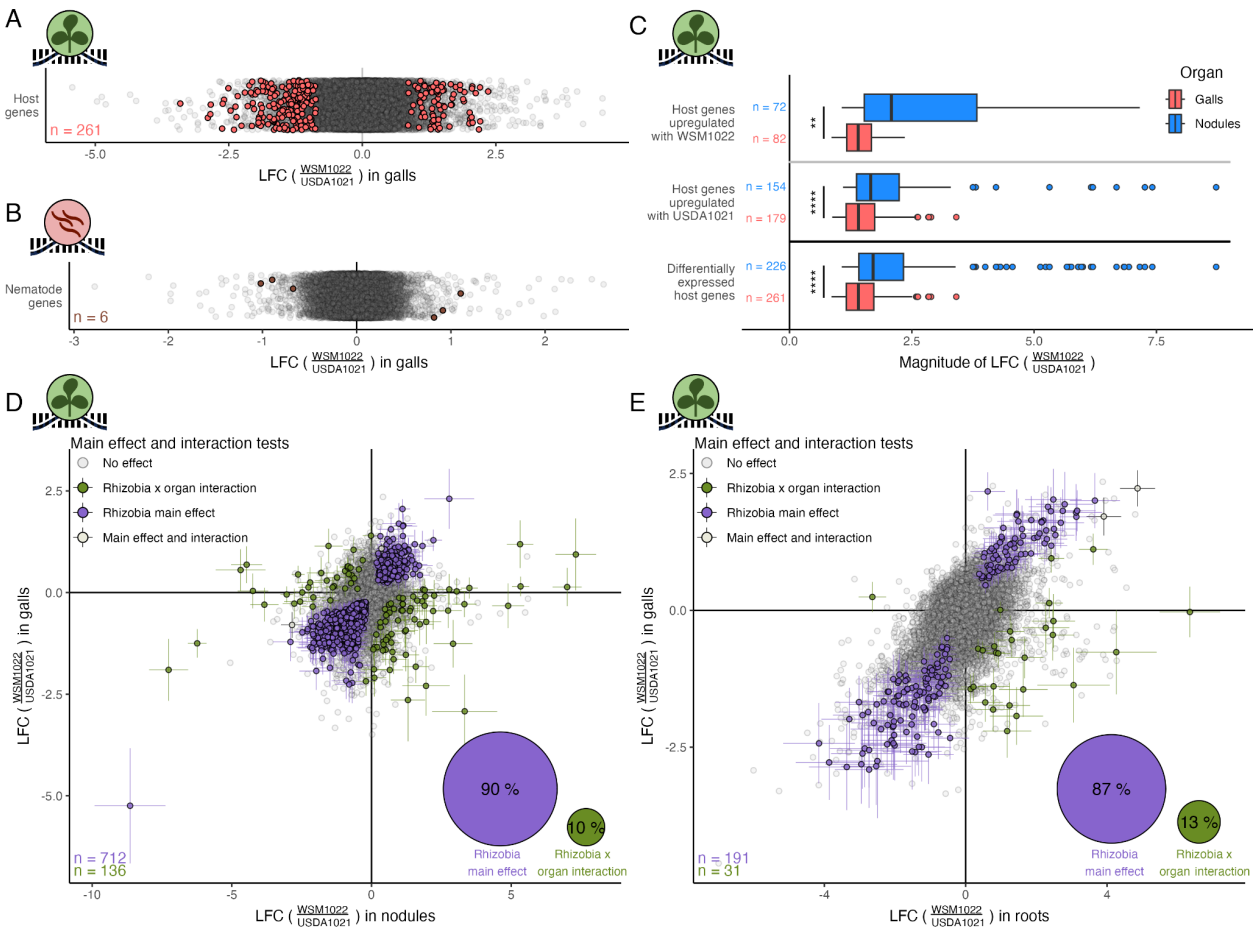

Figure 2: Rhizobia strain influences gene expression at the site of host-parasite interactions with largely similar responses in gene expression occurring in galls and nodules and in galls and roots. Log fold change (LFC) and standard error values were calculated using DESeq2. LFC values are the logarithm in base 2 of the ratio of normalized mean expression in hosts inoculated with USDA1021 divided by normalized mean expression in hosts inoculated with WSM1022. Genes with a significant log fold changes determined by likelihood ratio tests performed by DESeq2 are indicated with color in all panels and also with standard error bars in D and E. a) Of all host genes expressed in galls, 261 host genes were differentially expressed in galls in response to hosts being inoculated with different rhizobia strains. b) Of all expressed nematode genes, six nematode genes were differentially expressed in response to their host being inoculated with different rhizobia strains. c) Similar numbers of genes were differentially expressed in galls and nodules in response to different rhizobia strains resident in nodules (261 genes in galls and 226 genes in nodules), but the magnitude of differential expression was significantly higher in nodules than in galls. Similar trends are observed when comparing genes upregulated in hosts

with WSM1022 or USDA1021. Differences in magnitude were tested using the optimal pooled t-test (Guo and Yuan, 2017) ('n.s.' indicates no significance, * indicates p-value < 0.05, ** indicates p-value < 0.01, *** indicates p-value < 0.001, **** indicates p-value < 0.0001). d) Rhizobial differences produce largely similar responses in host gene expression in nodules and galls. Of all host genes expressed in nodules and galls, 712 (90%) showed a main effect of rhizobia indicating a similar response to rhizobia strain across organs and 136 (10%) showed an interaction effect of rhizobia strain and organ indicating a different response to rhizobia strain across organs. e) Rhizobial differences produce largely similar responses in host gene expression in roots and galls. Of all host genes expressed in roots and galls, 191 (87%) showed a main effect of rhizobia strain and 31 (13%) showed an interaction effect of rhizobia strain and organ.

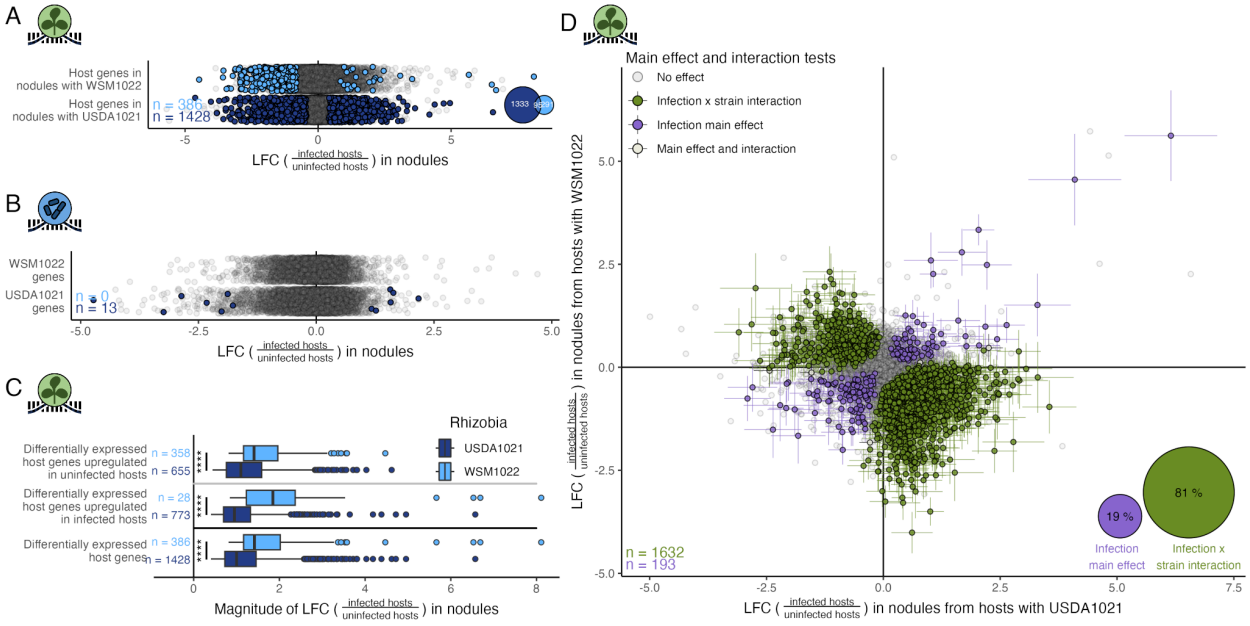

Figure 3: Nematode infection influences gene expression at the site of host-mutualist interactions in a largely strain specific manner. Log fold change (LFC) values were calculated using DESeq2 and are the logarithm in base 2 of the ratio of normalized mean expression in parasitized hosts divided by uninfected hosts. Genes with a significant log fold changes determined by likelihood ratio tests performed by DESeq2 are indicated with color and with standard error bars in D. a) Of all host genes expressed in nodules, 386 were differentially expressed in response to parasite infection in nodules with WSM1022 and 1,428 were differentially expressed in nodules with USDA1021. b) Of all expressed rhizobia genes, 13 USDA1021 genes were differentially expressed in response to their host being infected by nematodes. c) The magnitude of host

differential expression was significantly higher in nodules with WSM1022 than in nodules with USDA1021. Similar trends are observed when comparing genes upregulated or downregulated in parasitized hosts. Differences in magnitude were tested using the optimal pooled t-test (B. Guo & Yuan, 2017) ('n.s.' indicates no significance, * indicates p-value < 0.05, ** indicates p-value < 0.01, *** indicates p-value < 0.001, **** indicates p-value < 0.0001). d) Nematode infection produces a largely strain specific host response in the nodules. Of all the host genes expressed in nodules regardless of rhizobia strain, 1,632 genes (81%) showed an interaction effect of host nematode status and rhizobia strain whereas only 193 genes (19%) showed a main effect of rhizobia strain.

Table 1: Select gene lists

a. All genes differentially expressed in galls in response to rhizobial strain differences

Gene locus tag	Description (WormBase annotation)	Note
MhA1_Contig1719.frz3.gene12	β-lactamase HcpB-like inferred from homology	Nuclear localization signal sequence predicted by NLStradamus
MhA1_Contig253.frz3.gene16	predicted cylicin-2	Secreted protein predicted by SignalP 6.0; Shows similarity to presumed parasitism gene Minc00331 in *Meloidogyne incognita*
MhA1_contig338.frz3.gene4	predicted glutamate-5-semialdehyde dehydrogenase	
MhA1_Contig543.frz3.gene1	Predicted rhodopsin-like G-protein coupled receptors	Nuclear localization signal sequence predicted by NLStradamus
MhA1_Contig2902.frz3.fgene1	rhodopsin-like G-protein coupled receptors inferred from homology	
MhA1_Contig338.frz3.gene3	predicted amino acid kinase domain-containing protein	

b. All genes differentially expressed in rhizobia strain WSM1022 in response to host parasite infection

Gene locus tag	Description (similarity to RefSeq amino acid sequence)	Note
CDO29_RS04280	metalloregulator ArsR/SmtB family transcription factor	

CDO29_RS13585	Lrp/AsnC family transcriptional regulator	
CDO29_RS13595	homogentisate 1,2-dioxygenase	hmgA; annotated with GO:0004411 (homogentisate 1,2-dioxygenase activity)
CDO29_RS14670	phosphoserine transaminase	Annotated with GO:0004648 (O-phospho-L-serine:2-oxoglutarate aminotransferase activity)
CDO29_RS16270	hypothetical protein	
CDO29_RS20170	adenine deaminase	
CDO29_RS20175	NCS2 family permease	
CDO29_RS20670	CpaF family protein	
CDO29_RS27020	cupin domain-containing protein	
CDO29_RS27030	DHA2 family efflux MFS transporter permease subunit	Annotated with GO:0005886 (plasma membrane)
CDO29_RS27365	LuxR C-terminal-related transcriptional regulator	Annotated with GO:0006355 (regulation of DNA-templated transcription)
CDO29_RS31060	NADH:flavin oxidoreductase	
CDO29_RS31065	dimethylsulfoniopropionate lyase	

c. All genes showing similar response to rhizobial strain across all organ types. Non-putative and non-hypothetical annotations in bold.

Gene locus tag	Description (INRAE annotation)	Note
MtrunA17_Chr1g0199151	Putative protein	
MtrunA17_Chr1g0204471	Putative protein	
MtrunA17_Chr2g0293681	Putative endo-polygalacturonase	
MtrunA17_Chr2g0305481	Putative protein	
MtrunA17_Chr2g0323741	Putative protein	
MtrunA17_Chr3g0085071	Putative trichome birefringence-like domain, PC-Esterase	
MtrunA17_Chr3g0100161	Hypothetical protein	

MtrunA17_Chr3g0123961	Putative protein	
MtrunA17_Chr4g0035171	Putative glucose-1-phosphate adenylyltransferase	
MtrunA17_Chr4g0039821	**ATP-Binding-Cassette (ABC) transporter typeB (P-glycoprotein (MDR/PGP/ABCB) efflux/conditional auxin transporter)**	
MtrunA17_Chr4g0053411	Putative protein kinase RLK-Pelle-LRR-III family	
MtrunA17_Chr5g0413411	Putative endo-polygalacturonase	
MtrunA17_Chr5g0445161	Putative pirin domain, rmlC-like cupin domain superfamily, rmlC-like jelly roll	
MtrunA17_Chr5g0446771	Putative Red chlorophyll catabolite reductase	
MtrunA17_Chr7g0273321	**MtHPT8 (Histidine PhosphoTransfer protein 8)**	Isoform MtHPT1 is critical for coordination of developmental processes in many organs including nodules (Tan et al., 2019)
MtrunA17_Chr8g0364961	**MtTPS9 (Trehalose phosphate synthase 9)**	
MtrunA17_Chr8g0367111	Putative rhamnogalacturonan endolyase	
MtrunA17_Chr8g0382321	Putative major facilitator superfamily, MFS transporter superfamily	

Supporting Information

The following Supporting Information is available for this article:

Dataset S1: A comma-separated values file containing gene expression quantification data output from HTSeq-union for all genes in all organisms.

Dataset S2: A comma-separated values file containing gene expression status for all genes in all organisms.

Dataset S3: A comma-separated values file containing the results of all DESeq2 differential expression analyses for all *Medicago truncatula* genes.

Dataset S4: A comma-separated values file containing the results of all DESeq2 differential expression analyses for all *Ensifer meliloti* genes.

Dataset S5: A comma-separated values file containing the results of all DESeq2 differential expression analyses for all *Meloidogyne hapla* genes.

Dataset S6: A comma-separated values file containing all Gene Ontology overrepresentation analysis results.

Fig. S1: Primary component analysis of *M. truncatula* gene expression quantification data showing segregation of datapoints by organ type.

Fig. S2: Upset plot showing the intersection of *M. truncatula* gene lists produced by differential expression analyses comparing similar and dissimilar responses to rhizobial strain differences across different groups of organs.

Table S1: Select Gene Ontology terms that were enriched in various gene lists produced by differential expression analyses

Table S2: Constituents of the various fertilizer solutions used in this experiment

Table S3: Treatment type (nematode status and rhizobial strain) and host organ of origin for each sample

Table S4: Parameters for the DESeq2 likelihood ratio tests performed for each model

Table S5: Table showing the percentage of genes in each organ and treatment type that had a coefficient of variation within the treatment type that was greater than the coefficient of variation across all samples.

Methods S1: Differential expression analysis

Methods S2: Software and reference genomes

Methods S3: Fertilizer recipes and schedule

Methods S4: RNA extraction