The symbiotic capacity of rhizobium shapes root-associated microbiomes

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Research

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Abstract

Background: Root-microbiome interactions are of central importance for plant performance and yield. A distinctive feature of legumes in this context is that they engage in symbiosis with rhizobia, which are abundant in soils and include both symbiotic and non-symbiotic bacterial strains. If and how the capacity of rhizobia to form symbiosis modulates root-associated microbiomes are not well understood.

Results: We address this question by inoculating soybean (*Glycine max*) plants with wild type (WT) or a *noeI* mutant of *Bradyrhizobium diazoefficiens*. The *noeI* mutant produces a defective Nod factor and is thus compromised in its ability to establish functional symbiosis. Compared to soybean plants inoculated with WT rhizobia, plants inoculated with the *noeI* mutant showed a significant decrease in nodulation and root-flavonoid exudation, and exhibited strong changes in microbiome assembly in the rhizosphere and the rhizoplane. *NoeI* mutant-inoculated roots exhibited reduced diversity, co-occurrence interactions and a substantial depletion of beneficial microbes on the roots. The effects of the *noeI* mutation were absent in soils without plants, demonstrating that they are plant dependent. Complementation experiments showed that flavonoid supplementation is sufficient to restore recruitment of beneficial microbes.

Conclusion: The results illustrate that the capacity of a rhizobium to form microbial symbiosis dramatically alters root-associated microbiomes, most likely by changing root exudation patterns. The results of this study have important implications for our understanding of the evolution of plant-microbiome interactions in the context of plant-bacterial symbioses.

Background

The interactions between plants and microbial communities (including archaea, bacteria, fungi, oomycetes, and protists) impact host health, fitness and biogeochemical cycling [1–3]. The highly dynamic microbial communities that colonize the root-soil interface are particularly important in this context [4, 5]. Beneficial effects of root microbiomes include enhanced nutrient acquisition [6], disease suppression [7], plant immunity [8], abiotic stress tolerance [9], and improved adaptation to environmental variation [10]. Through the release of root chemicals, plants provide specific niches for root microbiomes which favor the survival and adaptation of specialized inhabitants [11]. Apart from plant chemistry, several other factors like soil type, host genotype, developmental stage, nutrient status and rhizosphere-compartmentalization are important determinants of root-associated microbiome assemblages [12, 13].

High genetic variation of different members of microbiome communities is frequent in nature [14]. While the impact of plant genetic variation on root-associated microbiomes is becoming more and more studied [15–17], much less is known about the importance of microbial genetic variation. Molecular mechanisms underlying heritable variation in individual microbes include spontaneous mutations, genomic rearrangements, slipped-strand mispairing and epigenetic mechanisms such as differential methylation [18, 19]. Each microbial species consists of genetically variable individuals [20]. In the

human gut, different individual microbial strains are genetically variable between different hosts and within a host over time [20, 21]. So far, microbial genetic variation has mostly been studied in host-pathogen interactions due to the interest in genetic variants enabling pathogens to evade the host immune [22]. For instance, a mutation of a site-specific recombinase gene in *Pseudomonas fluorescens* WCS365 causes reduced competitive colonization of root tips in tomato [23]. Studies have also uncovered links between microbial genetic variation and the production of secondary metabolites and biocontrol activity of rhizosphere bacteria [24, 25]. Evolution is apparent and fast in microorganisms [26], and root-microbiomes can thus be expected to be genetically dynamic. High-throughput sequencing studies often investigate the taxonomic or functional compositions of root-associated microbiomes [27], but do not approach the functional consequences of microbial genetic variation on the host plant. Genetic manipulation of individual strains can help to assess the importance of selected heritable traits in determining the composition and function of microbiomes in the context of root-microbiome interactions.

Over the course of evolution, legumes have developed mutualistic relationships with rhizobia, also called root nodule bacteria. This interaction involves forming root or stem nodules in which rhizobia convert atmospheric nitrogen (N_2) into ammonia (NH_3) that is used as nitrogen-resource for the legume. In turn, legumes supply photosynthates to their bacterial symbionts [28, 29]. Rhizobia-legume symbiosis is highly specific and widely diverse [30, 31]. For a given host, functional symbiosis between rhizobia is affected by the competitive ability of rhizobia and by environmental factors such as soil properties [32, 33]. Although it has recently been shown that the rhizosphere microbiome has a crucial regulatory role in shaping rhizobia-soybean symbiosis [34], little is known about the effect of rhizobia-host symbiosis on root-associated microbiomes.

The legume-rhizobial symbiosis begins with the secretion of flavonoids by roots of the plants. Plant flavonoid exudation is regulated by the presence of the bacteria, which specifically recognize the compounds by their NodD receptors [35, 36]. This in turn induces the bacteria to release Nod factors (NFs), which are signal molecules synthesized by their nodulation genes (*nod*, *nol*, and *noe*) [29, 30]. Most rhizobial nodulation genes are located on transmissible genetic elements such as the symbiotic plasmids or islands and can be transferred horizontally at high frequency within the species [37]. On the plant side, lysin motif (LysM) receptor kinases recognize and bind the compatible NFs, and then initiate the accommodation of the rhizobia and the nodule-formation process [29, 38]. Rhizobial nodulation genes and plant symbiotic signaling genes, including NF receptor genes and downstream common symbiotic signaling pathway (SYM) genes, which are shared with arbuscular mycorrhizal (AM) symbiosis, are necessary to establish the symbiotic relationship and nodule development [29, 30, 39]. Previous studies have shown that genetic variation in plant genes encoding the common SYM receptor of in *Lotus japonicas* [40, 41], *Glycine max* [42, 43], *Medicago truncatula* [44] and the non-leguminous *Oryza sativa* [45] drive the establishment of distinctive root-associated microbiomes. By contrast, the impact of microbial genetic variation such as in rhizobial nodulation genes on root-associated microbiomes remains unknown. This is particularly relevant in the light of recent findings showing that rhizobia

acquired the key symbiosis genes multiple times, and that the most recent common ancestor was able to colonize roots of many different plant species [46], begging the question if and how the evolution of symbiosis affects plant-microbiome interactions.

The common *nod* genes *nodA*, *nodB*, and *nodC* are responsible for synthesizing the core structure of the NFs and are necessary for most symbiosises, while other nodulation genes encode the specific modifications on the backbone of signaling compounds and have effects on host specificity [47]. The *noeI* gene is responsible for the methylation of the fucose moiety at the reducing end of the NFs [48]. Previous studies have found that *noeI* was not essential for *Sinorhizobium fredii* HH103 and *Rhizobium* sp. NGR234 nodulating several host plants [48, 49]. However, a recent study conducted on *B. diazoefficiens* USDA 110 found that *noeI* has a vital role in maintaining nitrogen fixation efficiency in soybean [50]. While nodulation phenotypes and host nitrogen status are known to have an impact on the structure of root- and shoot-associated microbiomes in soybean [42, 51], the effect of genetic variation in nitrogen-fixing symbionts is unknown.

In this study, we investigated the role of genetic variation in the *noeI* gene of *B. diazoefficiens* (strain USDA 110) in regulating the assembly of the soybean root-associated microbiota. We sampled five compartments (rhizosphere, rhizoplane, endosphere, nodules and unplanted soil) to determine the direct and plant-mediated effects of the *noeI* gene mutation on the composition and diversity of root-associated bacterial communities. Further, we investigated the potential role of plant flavonoids in triggering these effects. Our results reveal that *noeI* determines the composition of root-associated microbiota through plant-mediated effects such as increased flavonoid exudation. These findings shed light on the mechanisms underlying the relationship between root-microbe symbiosis and root-associated microbial communities.

Methods

Soil

Soil samples were collected from a perennially flooded paddy field located in Leshan, Sichuan Province, China (29.2593 N, 103.9403 E). Surface soil was collected at a depth of 0 to 20 cm through a “five points” sampling strategy in a 25 m × 25 m field. All soil samples were transported immediately to the laboratory on ice and stored at 4 °C. Plant residues, roots, and stones were removed, and the soil was drained well enough to pass through a 2 mm sieve. These soils were used in greenhouse batch experiments; they were chosen as they contain no native compatible rhizobia that can nodulate with *Glycine max* variety C08. The basic properties of the soil were: pH 5.3 (soil:water = 1:2.5); total carbon (TC), total nitrogen (TN), H and S contents, 1.95%, 0.16%, 1.01% and 0.05%, respectively; cation exchange capacity (CEC) 16.55 cmol kg^{-1}; dissolved organic carbon (DOC) and dissolved organic nitrogen (DON), 37.58 mg kg^{-1} and 2.93 mg kg^{-1}, respectively; exchangeable sodium (Na), potassium (K), calcium (Ca) and magnesium (Mg), 0.2497, 0.7898, 4.323 and 1.72 mg kg^{-1}, respectively. Cultivated soybean (*Glycine max*) variety C08 was used in this study.

Greenhouse experiment and symbiotic phenotype testing

The greenhouse experiment was of a complete factorial randomized block design (Fig. 1a) that consisted of two rhizobial genotype treatments and two planting patterns. The rhizobial genotype treatments included: 1) *B. diazoefficiens* USDA 110 wild type, isolated from soybean [52]; and 2) *B. diazoefficiens* USDA 110 *noeI* mutant, obtained from our previous study [50]. The two planting patterns were 1) planted with cultivated soybean (C08) and 2) intact soil without plants (unplanted). Planted and unplanted soils that were not inoculated with rhizobia were instead inoculated with sterile 0.8% NaCl (w/v) solution as negative control treatments. As such, the negative control of unplanted soil is also referred to as bulk soil.

Soybean seeds were selected for fullness and uniformity before being surface-sterilized in 95% ethanol for 30 seconds and then further sterilized with 2.5% (w/v) sodium hypochlorite (NaClO) solution for 3-5 minutes, after which they were rinsed seven times with sterilized deionized water. The sterilized seeds were germinated on 0.8% water-agar (w/v) plates in the dark at 28 °C for 36-48 h. Uniform germinated seedlings were selected and transferred into pots (10 by 12 cm height by diameter) containing 500 g of soil. Each treatment was inoculated with 1 mL of rhizobial culture (optical density at 600 nm [OD_{600}] concentration of 0.2, diluted with 0.8% NaCl solution), as described in our previous study [50]. Plants were grown in the greenhouse (day/night cycle 16/8 h, 25/16 °C and a relative humidity of 60%) and were harvested 45 days post-inoculation (dpi). Several symbiotic phenotypes were recorded for plants inoculated with the wild type and the mutant. Leaf chlorophyll concentrations were determined using a SPAD-502 meter (Konica Minolta, Osaka, Japan) [53]. Plant height, weight of fresh nodules and the number of nodules were measured after sampling and shoot and root weights were determined after being dried at 65 °C for 5 days. Nodule nitrogenase activity was measured using the acetylene reduction method as described in Buendiaclaveria et al. [54].

Sampling of unplanted soil, rhizosphere, rhizoplane, endosphere, and nodule

The method for sampling unplanted soil, rhizosphere, rhizoplane, endosphere and nodules followed the protocol described Edwards et al. [55] with the following modifications. Briefly, the plants were removed from each pot and the loosely attached soil on the roots was removed with gentle shaking, leaving the root-adhering soil layer (approximately 1 mm of soil). The soil collection steps were performed on ice. Firstly, the roots were placed in a sterile 50 mL falcon tube containing 30 mL of sterile pre-cooled PBS (phosphate-buffered saline) buffer (with pH 7.3-7.5) and vortexed for 15 s, and the turbid solution was filtered through a 100-μm aseptic nylon mesh strainer into a new 50-mL tube to remove root fragments and large sediments, followed by centrifuging for 5 min at 12,000 × g at 4 °C. The supernatant was discarded, and the soil washed from the roots was defined as rhizosphere soil, which was then frozen with liquid nitrogen and stored at -80 °C. For rhizoplane samples, the washed roots were transferred to a falcon tube with 30 mL PBS and sonicated for 30 s at 50-60 Hz twice. The roots were then removed, and the rhizoplane samples was collected by centrifugation at 12,000×g for 5 min at 4 °C and stored at -80 °C

until DNA extraction. The washed roots were cleaned and sonicated again as described before to ensure that all microbes were removed from the root surface. Two more sonication procedures using clean PBS solution were performed, and the sonicated roots were surface-sterilized in 70% (v/v) ethanol for 2 min and then in 2.5% (w/v) NaClO solution for 5 min, followed by washing with PBS solution for seven times. The root nodules were collected by separating them from roots using sterile blades. The roots were defined as endosphere samples and stored at -80 °C alongside the nodules. Unplanted soil samples were collected from unplanted pots approximately 2 cm below the soil surface and stored at -80 °C until DNA extraction.

DNA extraction, 16S rRNA gene sequencing, and analysis

Genomic DNA of each sample was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals, LLC., Solon, OH, USA) following the manufacturer's protocol. DNA concentration and purity were evaluated photometrically using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States). The extracted DNA was stored at -80 °C until further analysis. Primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the variable V4 region of the bacterial 16S rRNA gene. PCR conditions as follows: 94 °C, 5 min, 94 °C, 30 s, 52 °C, 30 s, 72 °C, 30 s, 72 °C, 10 min, 30 cycles. Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (New England Biolabs, MA, USA) following the manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Fisher Scientific, MA, USA) and Agilent Bioanalyzer 2100 systems (Agilent Technologies, Waldbronn, Germany). Finally, the library was sequenced on an Illumina_Hiseq2500 platform and paired-end reads of length 250 bp were generated (Guangdong Magigene Biotechnology Co., Ltd. Guangzhou, China). The resulting paired sequence reads were then merged, trimmed, filtered, aligned, and clustered to define the operational taxonomic unit (OTU) using USEARCH v.11.06 [56]. Briefly, sequences with ≥ 97% similarity were assigned to the same OTU by the UPARSE-OTU algorithm in USEARCH; and chimera detection was performed with VSEARCH 2.11 [57]. Putative chimeric sequences and singletons were discarded.

Root exudate collection and UPLC-MS/MS analysis

Full and uniform soybean seeds were surface sterilized and germinated as described above. To enhance root growth, germinated seedlings were transferred to sterile pots containing sterile vermiculite and grown in the greenhouse for 7 days under the same conditions as described above. At harvest, the soybean plants were pulled from their pots and washed to remove the vermiculite, then four plants were transferred to a 9-well sponge lattice placed in a glass jar (12.6 cm in height and 8.5 cm in diameter) containing 100 mL 25% (v/v) of sterile nitrogen-free Rigaud–Puppo solution [58]. The plant roots grew through the holes of the lattice into the nutrient solution. These hydroponics systems were inoculated with 4 mL of USDA 110 WT and *noel* mutant cultures as described above with 4 mL 0.8% NaCl added to the control samples. To provide an aerobic environment for rhizobia, oxygen was pumped into the

nutrient solution; each treatment contained three replicate hydroponics systems. The systems were incubated for 7 days in a climate-controlled growth chamber (day/night cycle 14/10 h, 28/16 °C and relative humidity of 60%). To check the sterility of the hydroponics systems, aliquot of 500 µL from each system was spread and cultured on tryptone-yeast (TY) medium plates. Soybean root exudates were collected by centrifugation at 10,000 rpm for 20 min (5 °C), filtered using a 0.25-µm cellulose nitrate filter and then stored at -20 °C until further analysis.

Eleven standard flavonoids (supplied by J&K or ANPEL) were determined during experiment: naringenin, hesperetin, genistein, daidzein, 7, 4'-dihydroxyflavone, apigenin, chrysin, luteolin, isoliquiritigenin, morin, coumestrol; deuterated genistein was used as the internal standard. The calibration curve was prepared by the serial dilution of a mixture of eleven standards by methanol with concentrations as follows: 50, 25, 10, 5, 1, 0.5, 0.1 µg/L. The internal standard was also added to all samples to achieve a final concentration of 10 µg/L. The calibration curve was obtained by plotting the peak area ratio (y) of the standard to the internal standard versus the ratio of their concentrations (x). The curve was fitted to a linear function with a weight of 1/nx (R^2 > 0.99), with “n” being the calibration level. The concentrations of the compounds in the sample were determined by their peak area ratio with the internal standard and were determined using the calibration curve. All standards and samples were filtered through a PTFE syringe filter (0.22 µm) and stored at -80 °C until further analysis.

The internal standard was added to each hydroponics culture (100 mL) to give a concentration of 10 µg/L after which the solution was passed through a Resprep C18 solid-phase extraction cartridge [Sep-Pak Vac 6cc (500 mg), Waters, USA]. Flavonoids were eluted by 10 mL methanol and then freeze-dried with liquid nitrogen. For quantification, samples were resuspended in 1 mL of 50% (v/v) methanol solution and 10 µL aliquots were injected into a Waters ACQUITY I-class UPLC coupled with Xevo TQ-XS Triple Quadrupole Mass Spectrometer in the electrospray ionization negative mode (Waters, USA). Liquid chromatography was performed on a 100 mm × 2.1 mm BEH C_{18} column with a particle size of 1.7 µm. The mobile phase consisted of solvent A (water) and solvent B (100% acetonitrile) and the flow rate was 0.3 mL/min. The optimized linear gradient system was as follows: 0–1 min, 5% B; 1–10 min, 35% B; 10–12 min, 95% B; 12–15.5 min, re-equilibrium to 5% B. The parameters of the mass spectrometer were as follows: capillary voltage 2.5 kV, cone voltage 80 V, desolvation temperature 600 °C, desolvation gas flow 1100 L/h, cone gas flow 250 L/h, nebulizer gas flow 7 bar, and collision gas flow 0.15 mL/min of argon. A multiple reaction monitoring (MRM) mode was employed for quantitative analysis. Mass spectral parameters were optimized for each analyte and are shown in Supplementary Table S1.

Impacts of the mixture of flavonoids on soil microbiome

To determine the effect of flavonoids on the structure of the soil microbiota, watery solutions were prepared containing a mixture of the eleven flavonoid standards according to the quantitative analysis of flavonoids secreted by soybean. The final concentration of daidzein was 1 µg/g, and the other ten flavonoids were added following their ratios to daidzein. From the soil described above, 100 g were

placed into pots and pre-incubated under the greenhouse conditions described above for one week to activate the soil microbiomes. 1 mL of the mixture solution was added into each pot twice a week for 4 weeks. The control treatment had the same volume of sterile water added; each treatment consisted of three replicates. All pots were watered twice a week during the incubation period. The soil samples were collected after incubation, with DNA extracted and the 16S rRNA gene sequenced and analyzed as described above.

Physicochemical characterization of soil

The soil physicochemical characteristics of each treatment were measured following the methods described by Bao [59]. Soil pH was measured using a suspension of soil and deionized water at a ratio of 1:2.5 (w/v). Soil total C, N, H and S contents were determined separately using an elemental analyzer (Flash EA 1112, Thermo Finnigan). DOC and DON were measured using a TOC analyzer (Multi N/C 3100, Analytik Jena AG). Soil exchangeable Na, K, Ca and Mg were extracted with 1 M ammonium acetate and measured by atomic absorption spectrophotometry (NovAA300, Analytik Jena AG). CEC was measured in a continuous colorimetric flow system (Skalar SAN++ System, Netherlands).

Statistical analysis

The resulting OTU table was normalized by the negative binomial model using the package *phyloseq* [60] in R (version 3.6.0). Weighted UniFrac [61] distances were calculated from the normalized OTU tables using the R package *vegan*, Principal coordinate analyses (PCoA) utilizing the weighted UniFrac distances to assess the differences in microbial communities between treatments. To measure the β-diversity significance, permutational multivariate analyses of variance (PERMANOVA) was conducted using the function *adonis* in *vegan* [62]. Shannon, Chao 1 and Fisher indices and the number of observed species were calculated using the function *diversity* in R package *vegan.* Kruskal-Wallis tests followed by Dunn's multiple-comparison test were performed to assess differences between treatments. The statistical analysis of taxonomic and functional profiles (STAMP) was applied to identify different species associated with rhizobial treatments [63]. To explore the correlation between microbial communities and environmental properties, weighted UniFrac distance-based RDA (db-RDA) and Variation partitioning analysis (VPA) were performed using the function *capscale* and *varpart* in the package *vegan*, respectively. To determine OTU enrichment in each treatment, a generalized linear model (GLM) approach using *edgeR* [55] was conducted. Microbial co-occurrence networks were constructed based on Spearman correlations among 300 dominant OTUs. The nodes in this network represent OTUs and links indicate potential microbial interactions. We adjusted all P-values of the correlation matrix using the Benjamini and Hochberg FDR controlling procedure. The indirect correlation dependencies were distinguished using the network deconvolution method [64]. The subnetworks for various compartments were induced based on OTU-presenting in corresponding samples. The cutoff for correlation value was determined through random matrix theory (RMT)-based methods [65]. Network properties were calculated with the *igraph* [66] package in R and visualized in Gephi 0.8.2 [67]. Fisher's Least Significant Difference

(LSD) test ($p < 0.05$) and Duncan multiple-comparison test ($p < 0.05$) using R package *agricolae* [68] were employed to analyze the difference of soybean symbiotic phenotypes and relative abundance of bacterial taxa, respectively. All figures in this study were generated using *ggplot2* [69] in R and OriginPro 2017.

Results

A mutation in noeI of B. diazoefficiens suppresses soybean nodule formation

Nodulation genes are essential for the establishment of symbiosis between legumes and rhizobia. To confirm the role of *noeI* in nodulation, we inoculated soybean roots with WT and *noeI* mutant of strain *B. diazoefficiens* USDA110 and then screened the roots for nodule formation (Fig. 1a). Inoculation with WT bacteria resulted in the formation of > 20 nodules a total weight of > 17 g, and a nitrogenase activity of > 45 $nmol*h^{-1}*mg^{-1}$ per plant (Fig. 1b-e). The mutation of rhizobial *noeI* significantly impaired the nodulation efficiency of USDA 110 in soybean, with significantly lower nodule numbers, nodule weight and nitrogenase activity (Fig. 1b-e). The number of nodules was reduced to < 2 nodules per plant, and the nitrogenase activity dropped to < 0.5 $nmol*h^{-1}*mg^{-1}$, thus confirming that *noeI* is not strictly essential, but promotes nodule formation in soybean. No nodules formed in plants grown on soils treated with sterile control solution, and no nitrogenase activity was detected, showing that native nodule-forming rhizobia are absent in the experimental soil (Fig. 1b). As plants were well-fertilized, plant height, leaf chlorophyll content and shoot and root dry weights did not differ among treatments (Fig. 1f-i), thus allowing us to assess the impact of *noeI*-dependent symbiosis on microbial communities independently of plant performance.

Compartment-specific modulation of microbial communities by noeI

To determine whether the *noeI* mutation altered the unplanted soil and/or soybean root-associated microbiomes, DNA was extracted from all compartments and bacterial community profiles were determined using amplicon sequencing of the 16S rRNA gene. After quality filtering and chimera removal, 6,302,405 sequences (mean, 68,504 per sample) were obtained from 92 samples and 5,667 microbial OTUs were identified at 97% sequence similarity. Alpha diversity was measured using Shannon, Chao1 and Fisher indices as well as with the number of observed OTUs (richness). Alpha diversity was highest in soil, rhizosphere and rhizoplane, intermediate in the root endosphere and lowest in root nodules (Fig. 2a, Supplementary Fig. S1a-c). In the rhizosphere and rhizoplane compartments, α-diversity was similar following WT- and control-inoculation, but significantly lower following inoculation with the *noeI*-mutant ($p < 0.05$) (Fig. 2a, Supplementary Fig. S1a-c). In the endosphere, α-diversity was higher in WT- and *noeI* mutant-inoculated samples than control samples (Fig. 2a, Supplementary Fig. S1a-c). No differences between treatments were found in unplanted soil and nodules (Fig. 2a, Supplementary Fig. S1a-c).

PCoA and PERMANOVA were performed using weighted UniFrac distances. Samples were separated by compartments (39.04% of variation explained, $p < 0.001$), and inoculation treatments (12.91%, $p < 0.001$) (Supplementary Fig. S1d, Supplementary Table S2). Furthermore, a significant interaction between

compartments and treatments was detected (18.34%, $p < 0.001$) (Supplementary Table S2). Treatment effects were detected in samples from the rhizosphere (39.88%, $p < 0.001$), rhizoplane (36.90%, $p < 0.05$), and endosphere (25.63%, $p < 0.03$; Fig. 2b). Microbial community composition in the rhizosphere and rhizoplane were comparable in WT-inoculated and control roots, but differed significantly in *noeI* mutant-inoculated roots. In the endosphere and unplanted soil, WT and *noeI* mutant-inoculated samples showed similar profiles, but were different from control samples. In the nodules, WT and *noeI* mutant-inoculated showed similar microbial profiles. These results were confirmed by PERMANOVAs (Supplementary Table S2).

Taxonomy analysis revealed differences in the relative abundance of taxa at class level between WT and mutant treatments in the rhizosphere and rhizoplane; most bacterial classes were less abundant in the samples inoculated with the *noeI* mutant when compared to those inoculated with the WT strain (Supplementary Fig. S2a, b). This relationship was not observed in unplanted soil (Supplementary Fig. S2a, b). *Ktedonobacteria*, *Planctomycetia*, *Caldilineae*, and *Sphingobacteria* classes differed significantly between WT and mutant treatments in the rhizosphere ($p < 0.05$) (Supplementary Fig. S2a). The relative abundance of the predominant bacterial classes was significantly different between unplanted soil and endosphere compartments ($p < 0.05$), but the differences between WT and mutant treatments were not as distinct (Supplementary Fig. S2c). As expected, a pattern of reduced microbial complexity and significantly different relative abundance was found in nodules compared to those of unplanted soil ($p < 0.05$) (Supplementary Fig. S2d). Taxonomic assignments at the family level using relative abundance revealed that the nodules in both treatments were dominated by bacteria belonging to the families *Bradyrhizobiaceae* and *Nannocystaceae* (Supplementary Fig. S2e). Furthermore, the 16S rRNA sequences of *B. diazoefficiens* USDA 110 mapped to the most abundant OTU (OTU_77) and accounted for 67.85% and 69.70% of the nodule profiles inoculated with WT and the mutant strain, respectively (Supplementary Fig. S2f). These results show that the mutation in *noeI* has compartment-specific effects on microbial communities.

NoeI affects niche differentiation in different rhizo-compartments

Enrichment analysis of OTUs using a generalized linear model confirmed differentiation of microbial communities across the rhizo-compartments. Compared to bulk soil, 49 bacterial OTUs mainly assigned to *Proteobacteria* (*Alpha*-, *Delta*-, *Beta*-, and *Gamma*-proteobacteria), and *Firmicutes* (*Bacilli*, *Clostridia*) were significantly enriched in the rhizosphere of soybean inoculated with WT strain (Fig. 3a). There was only one OTU (*Bacilli*) that was differentially enriched in the rhizoplane compared to the rhizosphere in the WT treatment. A total of 537 OTUs belonging to the phyla *Proteobacteria*, *Bacteroidetes*, *Planctomycetes*, *Actinobacteria*, *Firmicutes* and *Chloroflexi* were also enriched in the endosphere compared to the rhizoplane. Overall, 171 OTUs mainly consisting of *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Chloroflexi* were enriched in nodules compared to the endosphere (Fig. 3a). The pattern of microbial community differentiation across the compartments in *noeI* mutant-inoculated samples differed in the rhizosphere and rhizoplane (Fig. 3b). Specifically, there were 148 OTUs enriched in the rhizosphere relative to bulk soil, which mainly belonged to *Proteobacteria*, *Bacteroidetes*

and *Actinobacteria*. Compared to WT samples, the rhizoplane enriched a larger proportion of OTUs (27 vs 1) relative to the rhizosphere, which were mainly identified as members of *Alphaproteobacteria*, *Betaproteobacteria* and *Clostridia*. The microbial community differentiation between endosphere and nodule in mutant-inoculated samples was similar to WT-inoculated samples (Fig. 3b).

The STAMP method was performed to identify differences in taxonomic abundances between the WT and mutant treatments at the family level. Only *Pseudomonadaceae* were significantly enriched in the unplanted soil inoculated with the WT strain compared to that inoculated with the *noeI* mutant (Fig. 3c). A total of 11 families and 16 families were found to be significantly ($p < 0.05$) different between the inoculated soybean plants in the rhizosphere and the rhizoplane, respectively (Fig. 3c). Almost all of the differential families were enriched in samples inoculated with the WT strain, such as *Micromonosporaceae, Streptomycetaceae, Clostridiaceae, Geobacteraceae*, and *Sphingomonadaceae*. Strikingly, only one bacterial family in the endosphere samples showed a difference induced by the rhizobial treatments, with *Bradyrhizobiaceae* significantly enriched in the WT stain treatment (Fig. 3c). Finally, eight bacterial families were enriched in nodules inoculated with the WT strain; large differences were observed in *Burkholderiaceae* and *Sphingobacteriaceae* (Fig. 3c).

Noel **shapes microbial co-occurrence networks**

To determine whether the *noeI* mutation affects co-abundance patterns between bacterial taxa across the different rhizo-compartments, we first generated two full networks using bulk soil plus WT or bulk soil plus mutant samples using relative abundances of the 300 most abundant OTUs. We then constructed sub-networks for each rhizo-compartment from the corresponding full networks. In networks of WT- and *noeI* mutant-inoculated samples, the number of nodes and correlations in the sub-networks decreased from rhizoplane to nodule, with no differences between rhizosphere and rhizoplane (Fig. 4, Supplementary Table S3). Three dominant clusters were identified in all sub-networks. The first cluster consisted of *Bradyrhizobiaceae* and *Rhizobiaceae* families; the second cluster contained taxa from *Ktedonobacteraceae*, and the third cluster contained families from *Clostridiaceae_1.* This third cluster exhibited a greater number of connections in the sub-network from samples inoculated with the WT than that in the *noeI* mutant treatment (Fig. 4). The topological features of sub-networks differed in both rhizo-compartments and treatments (Supplementary Table S3). Specifically, the average degree of sub-networks decreased from bulk soil to nodule; the sub-network in bulk soil had the lowest modularity, diameter and number of clusters. The average path length, betweenness centrality and modularity of sub-network were highest in the nodule compared to the other rhizo-compartments (Supplementary Table S3). In the rhizobial treatments, the average degree, connectivity and number of clusters of sub-networks were higher in samples inoculated with the WT rhizobium than those inoculated with the mutant, whereas the average path length and diameter were lower in the WT treatment (Supplementary Table S3). Thus, the *noeI* mutation alters network topologies features of microbial co-occurrence in different rhizo-compartments.

Role of flavonoid exudates in noeI**-dependent effects**

To determine whether NoeI may modulate microbial communities by changing flavonoid exudation, we collected exudates from soybean plants following inoculation with WT and *noeI*-mutant of strain *B. diazoefficiens* USDA110 and analyzed them by UPLC-MS/MS. Eleven flavonoids were identified and quantified in soybean root exudates (Fig. 5a). Compared to control roots, WT-inoculation increased the exudation of the six most abundant flavonoids, including a 5-fold increase in daidzein ($p < 0.05$) (Fig. 5a). These increases were absent in exudates of plants inoculated with the *noeI*-mutant, whose flavonoid exudation profiles were similar to control roots (Fig. 5a).

In a next step, we explored the relationships between flavonoid exudation and rhizosphere bacterial community composition by weighted UniFrac distance-based redundancy analysis (db-RDA) (Fig. 5b). We also included several soil chemical factors into this analysis, including TC, TN, DOC and DON, pH, CEC. Although the majority of these soil chemical factors were highly correlated with each other (Supplementary Fig. S3a), daidzein was identified as the most significant factor that differentiated the rhizosphere microbial communities between the WT and mutant treatments. By contrast, soil exchangeable Mg^{2+} explained differences in microbiomes between rhizospheres of control and inoculation treatments ($p < 0.001$) (Fig. 5b). To further assess the contribution of soil exchangeable Mg^{2+} and daidzein to the diversity of microbial community in rhizospheres, variance partitioning analysis was applied; this metric indicated that soil exchangeable Mg^{2+} and daidzein explained 28.6% and 4.80% of microbial community variation, respectively (Fig. 5c). These results indicate that flavonoid exudation, and in particular daidzein, may be responsible for the differences in microbial community composition that are triggered by *noeI*.

To gain insight into the role of flavonoids in determining microbiome structure, we performed an incubation experiment using the same soil and supplemented it with a mixture of flavonoids, which contained 1 μg/g daidzein, and the other ten flavonoids were added following their ratios to daidzein, twice a week. After four weeks of incubation, we determined changes in bacterial community structure using amplicon sequencing. Alpha diversity was lower in the soil treated with flavonoids than that in the control treatment (Supplementary Fig. S3b). PCoA using weighted Unifrac distances indicated a closer separation compared between soil treated with flavonoids and rhizosphere inoculated with WT bacteria, with a peripheral distribution in the soil treated with water (control) and rhizosphere inoculated with mutant bacteria (Supplementary Fig. S3c). This was confirmed by PERMANOVA with 46.60% of variance ($p < 0.001$). STAMP analysis revealed that the families of *Burkholderiaceae* and *Sphingobacteriaceae* were significantly enriched in the soils treated with flavonoids compared to the control (Fig. 5d).

Discussion

In this study, a genetic approach (gene mutation) is used to demonstrate that the capacity of *B. diazoefficiens* to form a symbiosis with soybean has major effects on root-associated microbiomes through plant-mediated effects. Here, we discuss these findings in the context of root-microbiota interactions.

Our bacterial community sequencing approach confirmed a clear differentiation of bacterial community structure between unplanted soil, rhizosphere, rhizoplane, endosphere and nodule compartments, with a gradient of decreasing bacterial α-diversity from rhizosphere to endosphere and to nodules. This observation is consistent with previous studies in various plants of *L. japonicas* [40], soybean [70], pea [71], peanut [72] and rice [55]. Interestingly, disrupting symbiosis between *B. diazoefficiens* and soybean significantly reduced bacterial diversity in the rhizosphere and rhizoplane. This result is in line with a recent study on plant SYM mutants documenting a reduction of fungal diversity [41]. Together, these studies suggest that functional symbiosis favors a more diverse root microbiota. As it may change root exudate quality and thereby create opportunities for microbes to colonize the rhizosphere [51, 73].

In contrast to the root-associated compartments, there was no effect of the *noeI* mutation on the diversity and composition of bacterial communities in the absence of soybean plants. This shows that the effect is plant-mediated. As a gene involved in nodulation, *noeI* is only expressed under induction of flavonoids secreted from host plant [48]. Thus, the impact of the *noeI* mutation is indeed expected to be restricted to the interaction between the plant and *B. diazoefficiens.* Previous studies using SYM mutants in *M. truncatula* [44], *L. japonicas* [40] and soybean [42, 43], found significant effects of these mutations on root-associated microbial communities' assemblages. Even in non-leguminous plants such as *Oryza sativa*, a mutated SYM pathway gene (*CCaMK*) has been found to structure distinctive root-associated microbiomes, as reflected by enrichment in *Rhizobiales* and *Sphingomonadales* [45], thus complicating the interpretation of these results in the context of legume symbiosis. Our work strengthens the notion that the successful establishment of legume symbiosis has substantial knock-on effects on native legume root-associated microbiota. It is likely that these changes will impact plant performance and soil legacy effects, thus influencing plant productivity in nature and agriculture beyond the primary effect of the symbiosis. Understanding these consequences is an exciting prospect of this work.

Interestingly, the successful establishment of symbiosis resulted in an enrichment of OTUs that are associated with beneficial effects such as *Micromonosporaceae* and *Streptomycetaceae*. Previous studies revealed that *Micromonosporaceae* are widespread in nitrogen-fixing nodules of different legume species and that these organisms enhance symbiosis efficiency when being co-inoculated with rhizobia [74–79]. The *nifH*-like gene sequences obtained from the nodule endophytic *Micromonosporaceae* strains were similar to *nifH* from *Frankia*, a nitrogen-fixing actinobacterium that can develop symbiotic relationships with several woody dicotyledonous plants [75]. This similarity suggests that *Micromonospora* is capable of fixing nitrogen [74, 75]. *Streptomycetaceae* are reported to possess the ability to colonize the roots of *Pisum sativum* [80] and *M. sativa* [81] and they could also increase root nodulation efficiency and facilitated nutrient assimilation of their host plants. These findings suggest that a functional symbiosis with effective nodulation and nitrogen fixation in soybean may specifically promotesthe enrichment of beneficial microbes.

In our study, two members of *Clostridiales* (*Clostridiaceae*_1, *Clostridiales* incertae sedis IV) were also enriched in the samples inoculated with the WT strain, which is consistent with previous experimentation where *Clostridium* was enhanced by rhizobial inoculation [82]. *Bradyrhizobiaceae* were depleted in roots

of soybeans inoculated with the *noeI* mutant. This might be explained by a reduction in compatibility between the host plant and *Bradyrhizobium* caused by *noeI* mutation [50].

The bacterial families significantly enriched in nodules inoculated with WT strain and in soil supplemented with the flavonoid mixture are presented in Fig. 3c and Fig. 5c. These families included *Burkholderiaceae*, which contained some species able to form symbiosis with a certain legumes from the *Papilionoideae* subfamily [83, 84] and also some species dominate soybean nodule [85] or known as a plant growth-promoting strain in non-legume plants [86]. Our results are consistent with other studies that found also a depletion of *Burkholderiales* in the roots of *Lotus* symbiosis pathway gene mutants [40, 41]. Therefore, we suggest that the effective symbiosis promotes the enrichment of beneficial microbes. In contrast, we found a significant depletion of *Sphingobacteriaceae* and *Burkholderiaceae* in the rhizosphere and rhizoplane of plants inoculated with the WT strain. This might be a consequence of potential niche replacement as a compensatory effect following the exclusion of *Micromonosporaceae* and *Streptomycetaceae* from these compartments.

Network analysis, an approach to visualize and examine microbial abundance patterns, confirmed in the different sub-networks the gradient of decreased diversity observed from soil to root and nodule compartments. This is also reflected in the topological features of the sub-networks. We noticed higher average degree, connectivity and number of clusters, and lower average path length and diameter for OTUs in the networks of the WT strain treatment compared to those for the *noeI* mutant treatment. This observation is possibly linked with the enhanced diversity seen in in root-associated compartments of the WT strain treatment. For instance, the higher average degree indicates that there are more potential bacterial connections in samples inoculated with the WT strain than those in the *noeI* mutant inoculated samples (Average degree measures the number of direct co-occurrence links for an each OTU in the network [87]). Our results are consistent with other work showing that rhizobia inoculation lead to an increase in soybean rhizobacterial network connections [88]. The increased modularity and number of clusters from bulk soil to nodule supports the conclusion that the nodule compartment is a highly selective niche [40]. Co-occurrence networks also identified several microbial clusters, which were composed of plant growth-promoting microbes such as *Rhizobiaceae* and *Clostridiaceae_1* [89]. Specifically, the formation of larger and stronger clusters by family of *Clostridiaceae_1* in the sub-networks of samples inoculated with the WT strain than the mutant treatment might be the result of alpha diversity effects seen in the rhizosphere and rhizoplane compartments. Taken together, the network analysis suggested that a functional symbiosis enriches beneficial microbes and structures a more tighly connected bacterial network.

The flavonoids daidzein, coumestrol and genistein have been found in exudates of most soybean cultivars [90, 91]. Similarly, we found daidzein as the most abundant flavonoid secreted by soybean variety C08, followed by coumestrol and genistein. This is consistent with a previous study on root exudates under similar conditions [92]. Previous studies reported that the amounts of secreted flavonoids increased by inoculation with compatible symbionts or by treatment with Nod factors and were reduced by inoculation with *nodC* mutant rhizobium [35, 36, 93]. Accordingly, we found a significantly increased

exudation of most flavonoids when inoculating the WT strain. This has not been seen for the *noeI* mutant and is most likely due to the defective symbiosis.

Root exudates present a major organic carbon resource for soil microorganisms and drive the assembly of plant rhizosphere microbial communities. Specific compounds in exudates are thought to promote or suppress specific soil microbial members leading to the formation of distinctive root-associated microbiomes [92, 94]. Soybean secretes the flavonoids from the root surface to the surrounding rhizosphere. This is consistent with our results that the deficient symbiotic relationships, as mediated by the *noeI* mutation, affected the bacterial communities mainly in the rhizosphere and rhizoplane compartments. This is consistent with other studies that have revealed daidzein and genistein to shape soybean microbial communities [95, 96]. We also found that the exogenous supplementation of flavonoids affected soil microbiome diversity and significantly enriched beneficial microbes compared to the control-treated soil, which is consistent with a study of the relative abundance of *B. diazoefficiens* USDA110 increased in soybean treated with daidzein [92]. Redundancy analysis and variance partitioning analysis identified that soil exchangeable Magnesium (Mg^{2+}) and daidzein were significantly associated with a rhizosphere microbial shift. Mg^{2+} plays an important role in the metabolism of rhizobia and the development of nodules, because nitrogen-fixing requires ATP present as a Mg^{2+}-complex [97]. Thus, wild-type rhizobial strains require more magnesium from the soil than symbiosis defective ones. Several studies have suggested that exchangeable magnesium has an impact on soil microbial communities [98, 99].

Conclusions

In summary, our data point to the following model (Fig. 6): *noeI* promotes functional symbiosis, which promotes the secretion of flavonoids, which again shape the root-associated microbiome. We conclude that the symbiosis between legumes and rhizobia drives root microbiome assembly through plant-derived chemicals. The probable dual role of flavonoids in the establishment of symbiosis and the structuring of microbial communities likely results in a direct link between legumes, rhizobia and root associated microbiomes. Understanding the consequences of this interplay for plant performance and the evolutionary dynamics of symbiosis are exciting prospects of this work.

Additional files

Additional file 1: Figure S1. α-diversity (Chao 1, observed OTUs and Fisher indices) and β-diversity among different rhizobial treatments in unplanted soil, rhizosphere, rhizoplane, endosphere and nodule. **Figure S2**. Microbial community composition in rhizosphere (a), rhizoplane (b), endosphere (c) and nodule (d); (e) The relative abundance of the top abundant taxa in the nodule samples treated with WT and mutant rhizobia. (f) The relative abundance of *B. diazoefficiens* USDA 110 in nodule samples treated with WT and mutant rhizobia. **Figure S3**. (a) Correlation analysis of the environmental factors; (b, c) Within-sample diversity (Chao1) and β-diversity in soil inoculated with a mixture of flavonoids and the uninoculated control. **Table S1.** Mass spectrometry parameters and ion patterns of tested compounds. **Table S2.** The

effects of environmental variables on the microbial community assembly. **Table S3.** Topological features of co-occurrence networks.

Abbreviations

WT
wild type; N_2:Nitrogen; NH_3:Ammonia; NFs:Nod factors; LysM:Lysin motif; SYM:Symbiotic signaling pathway; AM:Arbuscular mycorrhizal; TC:Total carbon; TN:Total nitrogen; DOC:Dissolved organic carbon; DON:Dissolved organic nitrogen; CEC:Cation exchange capacity; Na:Sodium; K:Potassium; Ca:Calcium; Mg:magnesium; NaClO:Sodium hypochlorite; OTU:Operational taxonomic unit; TY:Tryptone-yeast; MRM:Multiple reaction monitoring; PCoA:Principal coordinate analyses; PERMANOVA:Permutational multivariate analyses of variance; STAMP:Statistical analysis of taxonomic and functional profiles; db-RDA:Distance-based redundancy analysis; VPA:Variation partitioning analysis; GLM:generalized linear model; RMT:Random matrix theory; LSD:Least Significant Difference

Declarations

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Availability of data and materials

The amplicon sequencing datasets were submitted to Genome Sequence Archive (GSA) and are accessible under the project accession number PRJCA002971.

Authors' contributions

J.X. and B.M. conceived and supervised the study. J.X., B.M., Y.L., W.C. and E.W. designed the experiment. Y.L. collected samples and extracted DNA. Y.L., B.M., K.Z. analyzed the data. Y.L. and Z.L. performed the root exudate collection and UPLC-MS/MS analysis. Y.L. and S.Y. performed visualization of the data. Y.L. wrote the first draft of the manuscript. B.M., W.C., E.S., K.S., L.H., M.E., E.W., Y. Z. and J.X. revised the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

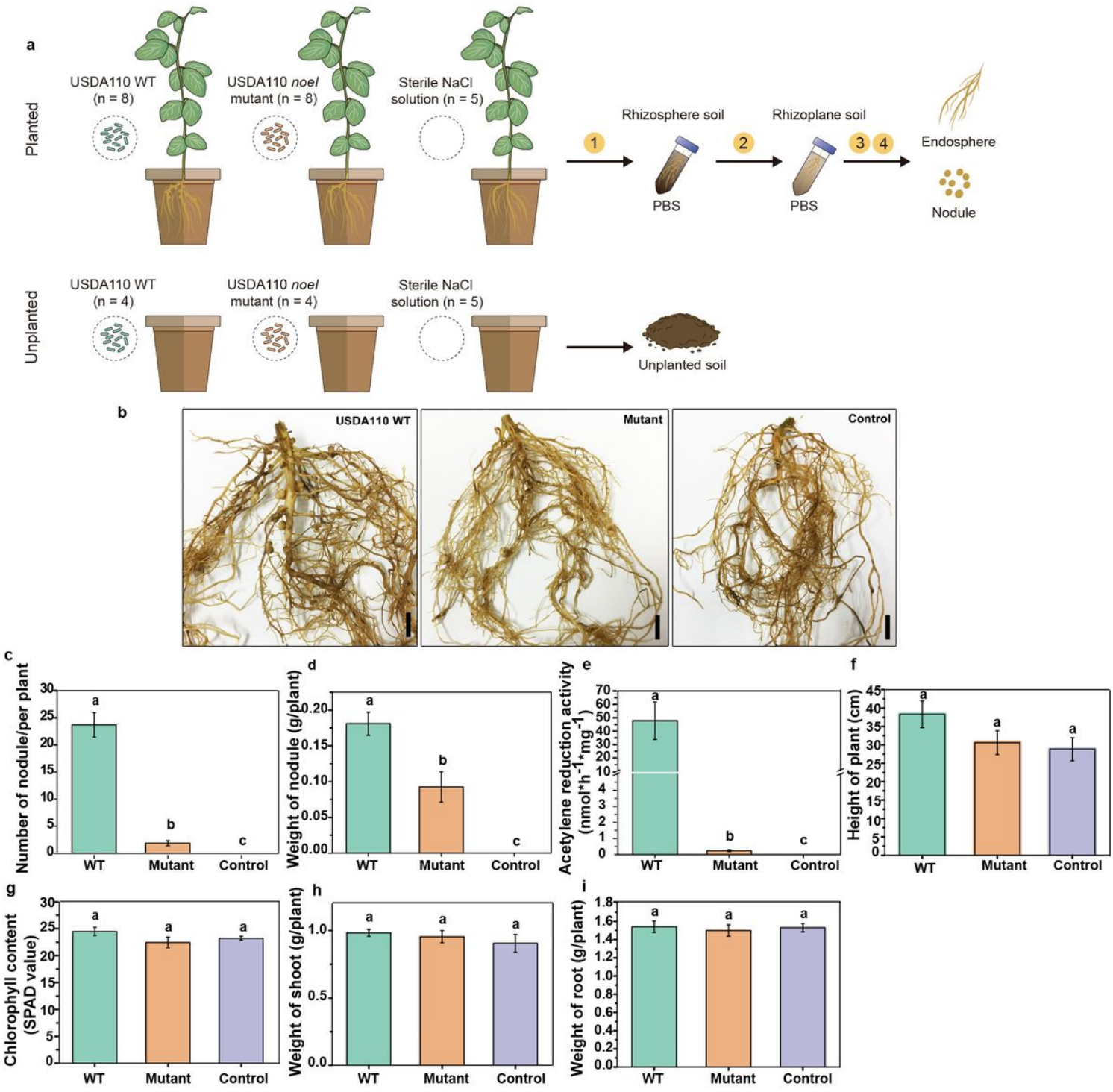

Figure 1

The experimental design and symbiotic phenotypes of soybean inoculated with rhizobia. (a) Soybean plants (Glycine max C08) were inoculated with B. diazoefficiens USDA110 WT or noeI mutants. Sterile 0.8% NaCl solution was used as control. The rhizosphere soil, rhizoplane soil, endosphere and nodules of were sampled 45 days post-inoculation (dpi). In addition, unplanted soil samples treated with the same treatments were collected at 45dpi. rhizosphere soil samples were collected by vortexed shaking and washing in PBS buffer, rhizoplane soil samples were collected from sonicating and washing. endosphere samples were obtained by surface-sterilizing, nodules were collected from the cleaned roots. (b) Images depicting the root system of soybean plants inoculated with the WT B. diazoefficiens or the noeI mutant or the control solution (scale bars: 1 cm); Scored nodulation phenotypes included (c) number of nodules per plant, (d) nodule weight, (e) nodule nitrogenase activity, (f) height of plant, (g) leaf chlorophyll content (SPAD), dry weight of shoot (h; g/plant) and root (i; g/plant). Means and standard errors are based on 16 scored plants; different letters indicate significant differences among treatments (LSD test, $p < 0.05$).

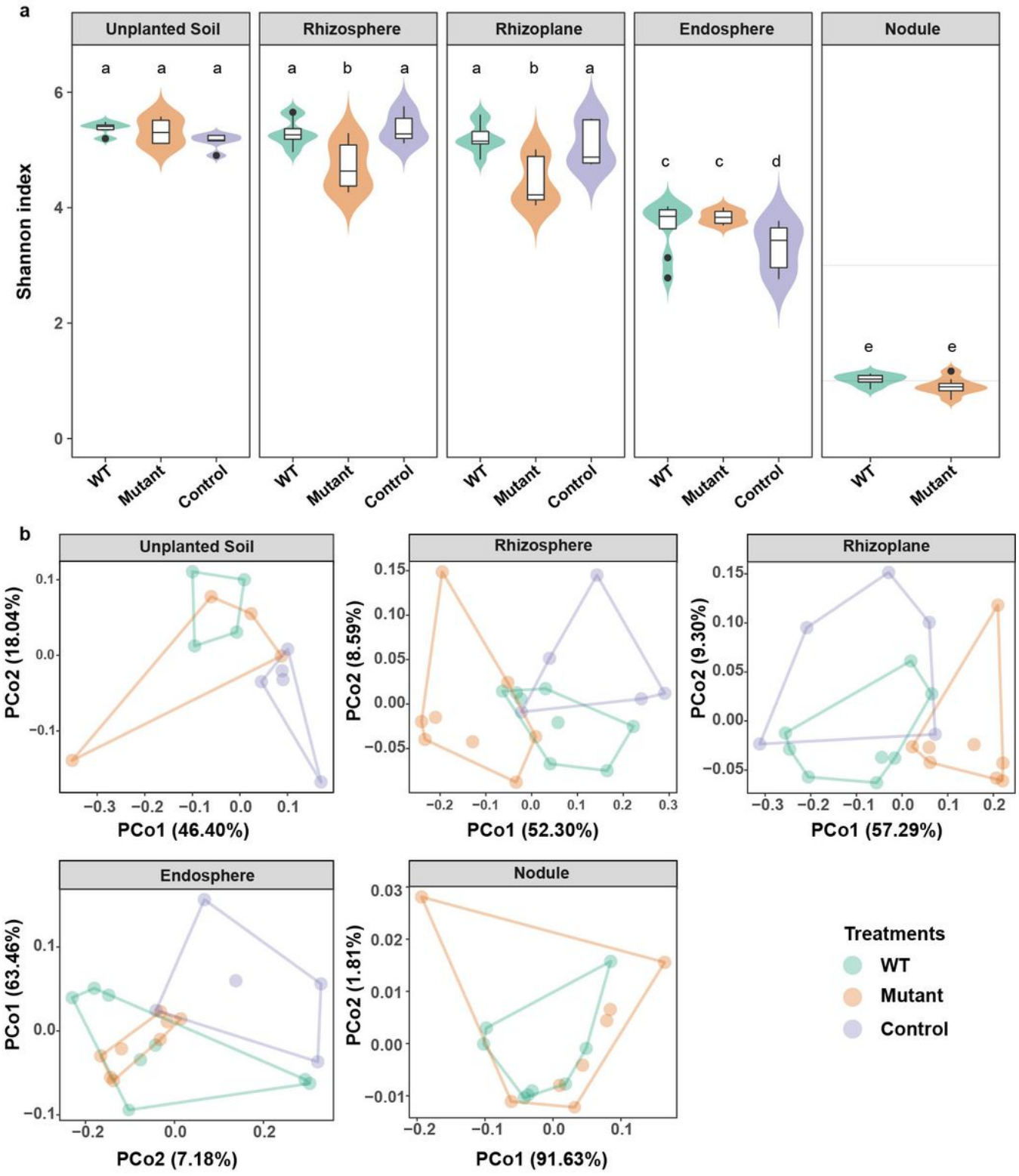

Figure 2

Compartment-specific modulation of microbial communities by noel. (a) α-diversity (Shannon index) between different rhizobial treatments in the unplanted soil, rhizosphere, rhizoplane, endosphere, and nodule compartments. Treatments are wild-type USDA 110 (WT), noel mutant (Mutant), not inoculated with rhizobia (Control). Different letters indicate significant differences among treatments (Dunn's multiple-comparison test; $p < 0.05$). (b) β-diversity principal coordinate analysis (PCoA; weighted UniFrac

distances) of unplanted soil, rhizosphere, rhizoplane, endosphere and nodule communities of soybean inoculated with wild type and mutant rhizobia, and of the control.

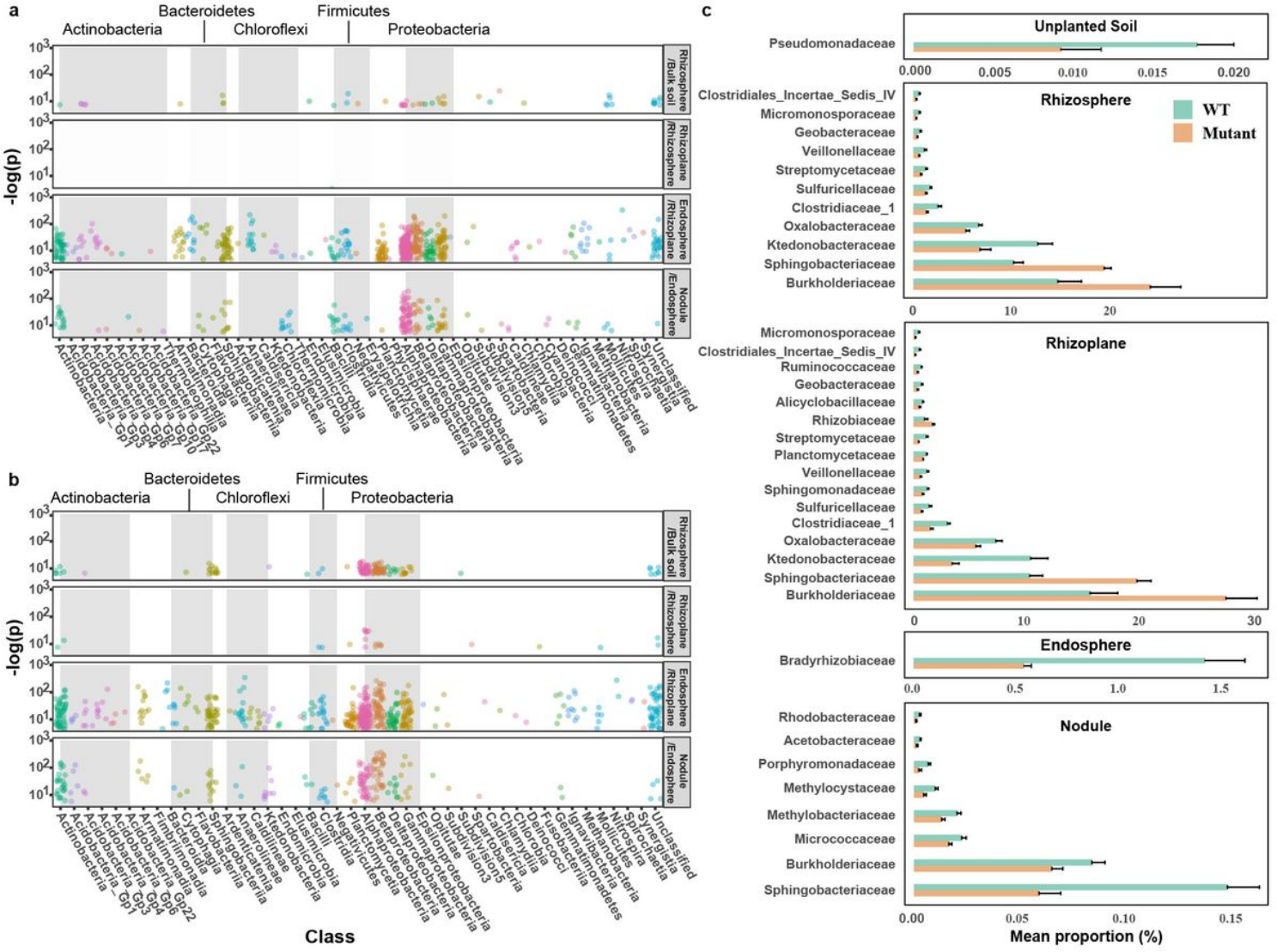

Figure 3

Noel affects niche differentiation in the rhizosphere. Bubble plots showing niche differentiation of rhizo-compartments in soybean inoculated with wild type (WT) USDA 110 (a) and noel mutant rhizobia (b). Compartment X/compartment Y (e.g. Rhizosphere/Bulk soil) represents the significantly enriched OTUs in compartment X relative to compartment Y ($p < 0.05$), bubble color indicates OTU taxonomic affiliation (class), and grey boxes indicate the OTU taxonomic affiliation (phylum). (c) Differences in taxonomic abundance between the WT and noel mutant treatments in unplanted soil, rhizosphere, rhizoplane, endosphere, and nodule samples at the family level (STAMP; Welch's t-test; $p < 0.05$).

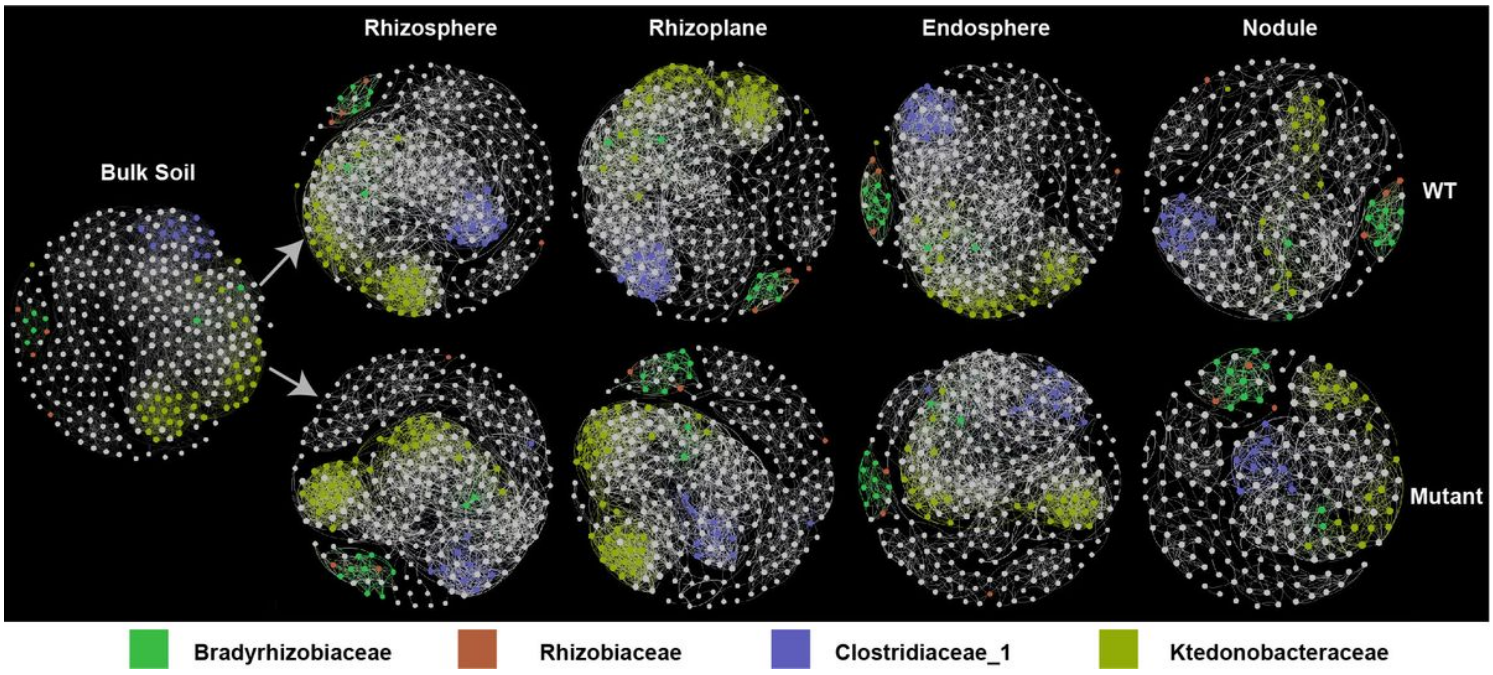

Figure 4

Co-occurrence of networks of rhizo-compartments in samples inoculated with rhizobia. The networks were constructed based on Spearman correlation analysis of taxonomic profiles; $p < 0.05$. Node size is proportional to degree; color indicates taxa (family); wild type (WT); noel mutant (Mutant).

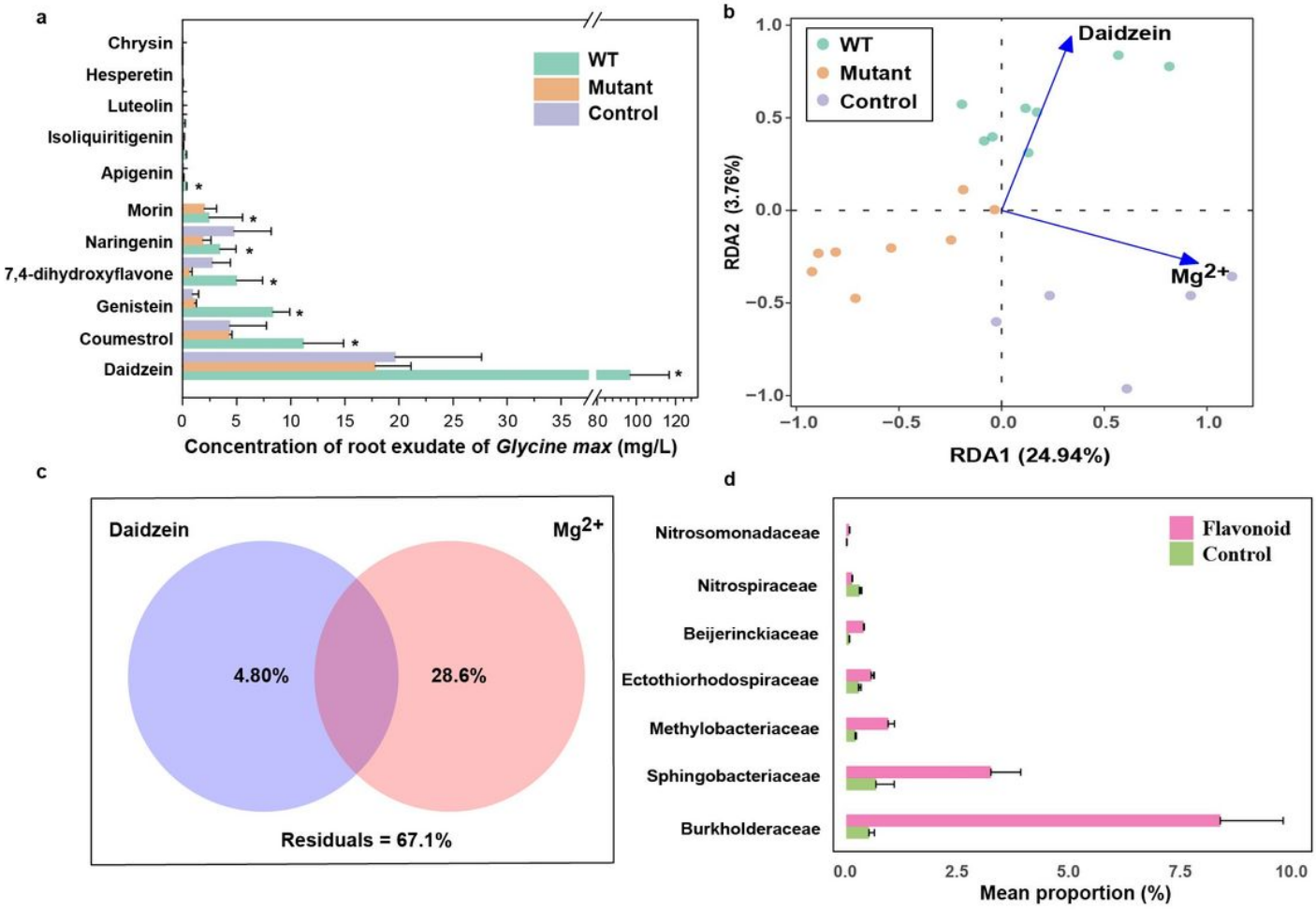

Figure 5

Role of flavonoid exudates in noel-dependent effects. (a) Flavonoid concentration in exudates collected from soybean inoculated with wild type (WT) and noel mutant (Mutant) rhizobia, and from uninoculated soil (Control); * indicates significant differences among treatments (LSD test, $p < 0.05$). (b) Redundancy analysis (RDA) of rhizosphere microbial community distribution and environmental factors, soil exchangeable magnesium (Mg2+). (c) The effects of dominant environmental factors on the structure of microbial communities in rhizosphere (VPA independent variance; value < 0 not shown). (d) Taxonomic abundance differences between soil supplemented with flavonoids and control (STAMP; Welch's t-test, $P < 0.05$).

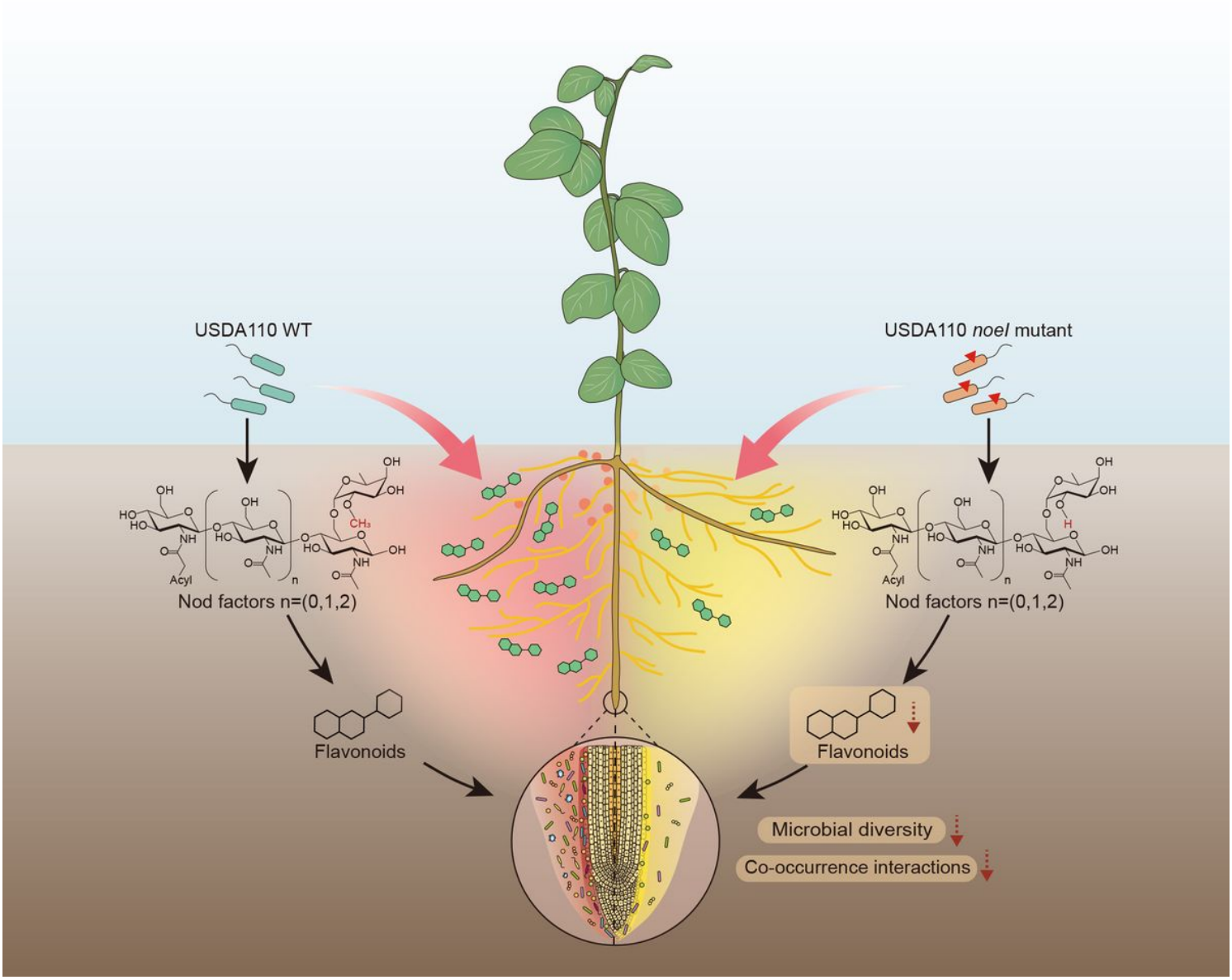

Figure 6

Proposed mechanisms underlying how rhizobium with mutated nodulation gene noel affects soybean root-associated microbiomes.

Supplementary Files

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