Plants release miRNAs in the rhizosphere, targeting microbial genes

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that control target gene expression, through sequence complementarity. Their roles in plants vary from regulating developmental processes to responding to abiotic and biotic stresses. Recently, small RNAs have been shown to play important roles in cross-kingdom communication, notably in plant-pathogen relationships. Plant miRNAs were even shown to regulate gene expression in the gut microbiota. Thus, we hypothesised that the same process happens in the rhizosphere which contributes to shaping plant microbial communities. To explore these questions, we performed small RNA sequencing in search of miRNAs in the rhizosphere of evolutionarily distant plants, *Arabidopsis thaliana* and *Brachypodium distachyon*. This revealed the presence of specific and shared rhizospheric plant miRNAs, which were all absent in unplanted soils. A subset of these miRNAs were also detected inside rhizospheric bacteria, but were missing in bacteria from unplanted soils, suggesting bacterial uptake of surrounding plant miRNAs. Furthermore, an *in silico* analysis indicated potential targets of these rhizospheric miRNAs in plant-associated bacterial genomes. To examine the function of these miRNAs, *A. thaliana* mutants, affected in their miRNA and/or siRNA (small interfering RNA) biosynthesis, were grown. Their rhizospheric microbial communities were significantly disrupted in comparison with wild-type plants. This work makes an important contribution to the field of rhizospheric plant-microbe interactions and offers some significant insights into the potential of plant miRNAs for microbiota engineering.

Introduction

The rhizosphere, defined as the portion of soil around the roots under the influence of plants, is a melting pot of soil microorganisms: from the typical trio of bacteria, fungi and archaea, to protists and nematodes. As the interface between plant and soil, the rhizosphere is the stage of many crucial plant-microbiota interactions. The rhizospheric microbiota plays many essential roles in maintaining plant health, such as provision of nutrients, defence against pathogenic invaders and protection during abiotic stresses[1]. Given the importance of these microbial communities in growing robust plants, many studies have examined the parameters that govern the composition and function of these communities, with the hope of optimising our agriculture through microbiota engineering[2]. The main factors that influence the dynamics of these microbial communities are either from the environment, from the plant or from the microorganisms themselves [3,4]. To fully understand the rhizospheric microbiota, all these factors have to be considered, however, the plant component is often emphasised resulting in considerable amounts of data on the capacity of plants to modify the surrounding microbial communities. Mainly formed by the existing soil microorganisms and marginally by the seed-borne microbiota[5–7], the root microbiota is influenced by factors depending on the compartment, *i.e.* endosphere, rhizoplane or rhizosphere, creating corresponding sub-microbiotas. The rhizospheric microbiota is then finely tuned by rhizodeposits, especially root exudates, depending on the plant species, genotype, root morphology, developmental stage and environmental factors, but is also heavily dependent on the biogeography of soil microorganisms[3]. Roots exudates are of diverse nature such as sugars, peptides, amino acids, nucleic acids, nucleotides, fatty acids or secondary metabolites[8].

Although many studies have clarified the role of these exudates in shaping the rhizomicrobiome, very few have focused on root-secreted nucleic acids. Yet, it is known that plants excrete extracellular DNA (exDNA) *via* their border cells, which are released from the root tip and are a great proportion of rhizodeposits[9]. This exDNA forms a matrix that functions as a trap surrounding the root mucilage to attract and immobilise pathogens[10], somewhat shaping the microbiota. Likely, we hypothesised that small RNAs, notably microRNAs (miRNAs), are an important class of nucleic acids that can be exuded through the roots and serve as a tool for engineering the microbiota of the rhizosphere[11]. These regulatory ~21nt non-coding RNAs are found in *Arabidopsis* roots and over half of them are expressed in a compartment-specific manner[12]. Interestingly, whilst no previous studies provided evidence of miRNA secretion in the rhizosphere, many miRNAs are enriched at the root tip, in the early meristematic zone[12]. This coincides perfectly with the fact that the root tip is a hotspot for active bacteria and is probably a region where plants concentrate their selective power[13], potentially through root-secreted miRNAs.

Small RNAs have been increasingly investigated in the context of plant-microbe interactions. Several studies have concluded that plants export small RNAs into pathogens: for example, cotton plants were shown to send miRNAs to the fungal pathogen *Verticillium dahliae* in order to silence virulence genes[14] and *Arabidopsis thaliana* delivered small RNAs *via* vesicles to the attacking *Botrytis cinerea*, inhibiting virulence[15]. The reverse is also true, pathogens are able to produce miRNAs or miRNA-like RNAs (milRNA) to counteract the defence of plants: for example, the fungal pathogen *Puccinia striiformis* produces a milRNA capable of silencing the pathogenesis-related 2 (PR2) gene in wheat[16] and the parasitic plant *Cuscuta*

campestris haustoria accumulates miRNAs capable of silencing *Arabidopsis* mRNAs during parasitism[17]. These small RNAs mediate cross-kingdom communication and are often the result of the famous arms' race between plants and fungal pathogens[18].

Research in plant-microbe interactions has tended to focus on pathogens, whereas studies in the animal kingdom have demonstrated that miRNAs play essential roles in shaping the microbiota as a whole. This is the case regarding human and murine intestinal miRNAs which regulate gut bacterial gene expression and growth, thus shaping the gut microbiota[19]. Interestingly, miRNAs from edible plants, conveyed in exosome-like nanoparticles, were shown to be preferentially uptaken by gut bacteria and able to regulate bacterial gene expression[20]. Regarding the role of miRNAs in the gut microbiota and the commonalities between the intestine and the rhizosphere[21], it seems reasonable that root-secreted miRNAs might be one of the important factors that shape the biodiversity and activity of this interacting rhizomicrobiome. In this paper, we explored this hypothesis, knowing that the existence of such miRNAs in the rhizosphere, prior to this study, was unheard of.

Here, we report, for the first time, the detection and identification of plant miRNAs in the rhizosphere of both *Arabidopsis thaliana* and *Brachypodium distachyon*, using small RNA sequencing. We then identified some of these miRNAs inside of bacterial cells, isolated from the rhizosphere of *Arabidopsis*, indicating that rhizobacteria may uptake surrounding plant miRNAs. These findings led us to search for potential miRNA targets in the genomes of bacteria and fungi isolated from the rhizosphere of *Arabidopsis thaliana*. The potential microbial engineering role of plant small RNAs, namely miRNAs and siRNAs (small interfering RNAs), was explored using *Arabidopsis* mutants, with impaired small RNA biosynthesis. The rhizospheric microbial communities of these mutants were disrupted in comparison with wild-type plants suggesting that miRNAs may play an essential role in controlling the rhizomicrobiome.

Results

Plant miRNAs are present in the rhizosphere of evolutionarily divergent plants

The potential presence of plant miRNAs in the rhizosphere was investigated by extracting rhizospheric RNA and performing small RNA sequencing. Two phylogenetically distant species were used in this experiment: the model dicotyledon, *Arabidopsis thaliana* and the model monocotyledon, *Brachypodium distachyon*. An average of 34,119,348 and 33,766,319 raw sequences per sample (**Table S1**) were obtained from the rhizospheres of *A. thaliana* and *B. distachyon*, respectively, and processed with the bioinformatic pipeline described in *Material and Methods*. After size filtration, an average of 18,351,294 (*A. thaliana*) and 18,468,688 (*B. distachyon*) sequences remained and 12-15% of these sequences were mapped against the reference genome of the corresponding plant, in rhizosphere samples, and 7-12% in unplanted soil samples. Amongst the annotated miRNAs, many were represented by a small number of reads, however they were consistently in higher numbers in the rhizosphere than in unplanted soil. To further confirm this result, the DESeq2 function was used and raw data was normalised within the function to compare differentially abundant miRNAs between rhizosphere and unplanted soil samples. In *A. thaliana* samples, 141 ath-miRNAs (mapped on *A. thaliana*'s genome) were detected, of which 29 were significantly more abundant in the rhizosphere as compared with unplanted

soil (**Figure 1**). The most abundant miRNAs in the rhizosphere were ath-miR158a-3p, ath-miR161.1 and various members of the miR159 family. In *B. distachyon* samples, 20 bdi-miRNAs (mapped on *B. distachyon*'s genome) out of 105 miRNAs were significantly more abundant in the rhizosphere compared with unplanted soil (**Figure 2**). Particularly, bdi-miR159b-3p was highly present, along with bdi-miR7757-5p and members of the bdi-miR396 and bdi-miR167 families. The normalised counts of these significantly abundant miRNAs, detected in the rhizosphere of these two plants, were higher in comparison with the counts from unplanted samples (**Figures S1 and S2**), confirming that the majority of plant miRNAs detected were in the rhizosphere samples, with the exception of ath-miR5026. Amongst these rhizospheric miRNAs, some were common to both *Arabidopsis* and *Brachypodium*, and undetected in unplanted soils, suggesting a potential conserved core of miRNAs secreted in the rhizosphere: miR159b-3p, miR166a-b-c-d-e-f, miR167b-d and miR396a-b (1-2 mismatches). We independently replicated this experiment in a different laboratory using the same plant species but different growth substrate and RNA extraction method, and retrieved highly coherent results.

Arabidopsis miRNAs are internalised in soil bacteria

Bacterial cells were isolated from the rhizosphere of 1 month-old *A. thaliana*, washed and their RNA content was extracted and sequenced to identify potentially internalised plant miRNAs. The intra-bacterial putative miRNAs were mapped against *A. thaliana* TAIR10.1 genome and a total of 97 ath-miRNAs were identified. These miRNAs were mainly detected in bacteria isolated from the rhizosphere and 10 ath-miRNAs were significantly more abundant in rhizospheric bacteria compared with bacteria from unplanted soil (**Figure 3**). To confirm the plant origin of these miRNAs, their sequences were searched for in 3,837 soil bacterial genomes[22], of which 1,160 were of bacteria isolated from plants. No matches were found, confirming that the detected miRNAs could not be produced by the bacteria themselves.

Disruption of small RNA biosynthesis in *Arabidopsis thaliana* affects rhizospheric microbial communities

To investigate the role of plant small RNAs on the rhizospheric microbiota, *A. thaliana* mutants, with disturbed miRNA and/or siRNA biosynthesis pathways, were grown and their rhizospheric microbial communities analysed by 16S rRNA gene and ITS region amplicons sequencing. These mutants are described in *Material and Methods*. To homogenise our results across samples and to reveal more realistic quantitative information, reads were normalised using the number of copies per sample, of 16S rRNA gene or ITS amplicons, measured by qPCR (**Figure S5**).

First of all, the general composition of the root and rhizospheric microbial communities was explored by comparing the taxonomic profiles of mutants and wild-type (WT) plants. At the phylum level, bacterial communities were quite similar between samples (**Figure 4**), however, WT plants tend to contain a lower phyla diversity in their root and rhizospheric communities. The fungal taxonomic profiles varied more across mutants and WT plants (**Figure 5**). Notably, root and rhizospheric communities from *dcl1-2* and *RLT1* mutants were distinguishable due to their high proportions of Chytrids, in comparison with other mutants and WT plants. This observation was extended to the distant rhizosphere of *RTL1* mutants.

To visualise the similarities of these mutants and WT rhizospheric communities, a Principal Coordinate Analysis (PCoA) was performed, using a weighted UniFrac distance matrix, which takes into account the abundance of each Amplicon Sequence Variant (ASV). Some observations are common to both bacterial and fungal communities (**Figures 6 and 7**, respectively). Firstly, PERMANOVA tests indicate that plant type, *i.e.* mutants or WT, explains a significant part of the variance seen in these microbial communities ($P<0.05$ for bacteria in roots and close rhizosphere; $P<0.001$ for other analyses). Secondly, *ago1-27* and *RTL1myc* mutants' communities are more similar to unplanted soil communities than those of WT plants.

Alpha-diversity indices, of which the number of observed ASVs, were established across our samples, revealing that bacterial diversity was significantly greater in mutant roots and close rhizospheres, compared with WT (**Figure 8**). There was also a significantly higher number of observed fungal ASVs in the distant rhizospheres of mutants compared with WT (**Figure 9**).

Plant rhizospheric miRNAs potentially target bacterial genes

In silico analyses were performed to seek out potential plant miRNA targets in bacterial genomes, using our bioinformatic pipeline. From the 428 known ath-miRNAs, 1,897,464 potential target sequences were found in the 3,837 soil bacterial genomes described by Levy, *et al*., in 2018. After selecting the 141 genomes of bacteria specifically isolated from the rhizosphere of *Arabidopsis* and searching for the targets of the 10 ath-miRNAs found in rhizospheric bacteria, the number of target sequences decreased to 2,101 (**Table S3**). As described in *Material and Methods*, target regions were positioned relative to the nearest coding DNA sequence (CDS), and targets found too far from any CDS were discarded due to their insignificant effect on mRNA translation. Two main regions were targeted: inside the CDS or far away from any CDS on the same strand (category "other"; **Figure S6**). The following analysis focused only on targets in identified CDSs, since this is where miRNAs are likely to exert a direct effect on gene transcription. When the other regions were removed, the 10 ath-miRNAs found in rhizospheric bacteria had a total of 1,189 potential targets, in 139 distinct *Arabidopsis* rhizospheric bacterial genomes. Interestingly, these ath-miRNAs were also predicted to target 961 sequences in non-plant-associated bacterial genomes. In both categories of bacteria, amino acid transport and metabolism was the main class of functions targeted by the intra-bacterial ath-miRNAs (**Figure 10**). However, the number of targets with these functions was significantly higher in *Arabidopsis* rhizospheric bacteria ($P=0.008$, **Table S4**). Some miRNAs targeted specific amino acid biosynthesis, for example, miR159c seemed to mostly target serine related mRNAs, whereas miR158a-3p targeted isoleucine, leucine and valine biosynthesis genes (**Figure S7**). Another highly targeted class of functions was related to energy production and conversion. The taxonomy of the targeted bacteria was investigated, and counts were normalised depending on the amount of each available taxa in the database. Within the rhizobacteria category, the most targeted families were *Bacillaceae* and *Paenibacillaceae* (**Figure S8**). Targeted families within non-plant-associated bacteria were more diverse: *Moraxellaceae*, *Bacillaceae*, *Pseudomonaceae*, *Flavobacteriaceae*, etc… In summary, plant miRNAs internalised in rhizobacteria are predicted to mainly target genes related to amino acid transport and metabolism, particularly in bacteria from the *Bacillaceae* and *Paenibacillaceae* families.

Methods

Detection of plant miRNAs in the rhizosphere

Arabidopsis thaliana (Col-0) and *Brachypodium distachyon* (Bd21-3) were planted in triplicates and grown for a month, alongside unplanted soils. The rhizospheres and control soils were sampled and flash-frozen with liquid nitrogen. RNA was then extracted from 2g of soil using MOBIO RNA Power soil kit, eluted in 100µL, following the manufacturer's guidelines. RNA quality was inspected with Nanodrop and 1% agarose gel and was then sent for sequencing on an Illumina HiSeq 2500 in single read mode (1x50bp) at the Centre d'expertise et de services Génome Québec (Montreal, Canada). Reads were trimmed using Trimmomatic[23] (v0.39), using the following Illumina clip settings :5:10:4. Common contaminants, such as Illumina adapters and PhiX spike-in sequences, were removed using the BBDuk tool (bbmap v38.11, https://github.com/BioInfoTools/BBMap). Sequences were then filtered by size: those with a length between 18bp and 27bp were kept. Selected reads were mapped against either *A.thaliana* (TAIR10/GCA_000001735.1) or *B.distachyon* (bd21/GCF_000005505.1) reference genomes, which are the same as used in miRBase[24]. Using BEDTools v.2.29.2[25] with -F 0.95 argument, a number of reads aligned inside miRNA coordinates were determined, meaning that at least 95% of each read had to belong inside the miRNA coordinates to be considered a valid hit. These assigned miRNAs which were mapped against the reference database were kept for abundance estimation across samples. The remaining reads were normalised using edgeR, with Reads Per Kilobase Million (RPKM).
A differential analysis was performed using DESeq2[26] to compare the abundances of each miRNA between 2 conditions (unplanted soil VS rhizosphere). For each miRNA, raw counts are normalised by calculating a pseudo-reference across all samples (geometric mean) and dividing counts of each sample by the pseudo-reference resulting in a ratio. For each sample, the median of ratios is considered as a normalisation factor or size factor. Normalised counts are calculated by dividing raw counts by the size factor. Our data set was composed of many zeros, so the geometric mean was calculated using a zero-tolerant variant of the formula to estimate size factors. A parametric fit along with a Wald test, using manually adjusted *P*-values <0,05 (Benjamini-Hochberg), provided a robust analysis of differentially abundant miRNAs.

Detection of plant miRNAs in rhizospheric bacteria

A.thaliana were grown for a month, then the rhizosphere was sampled by shaking off the loose soil and retaining the remnant soil around the roots. Bacteria were isolated from 5g of rhizospheric soil, using a density gradient. Samples were added to 30mL of 0,2% sodium hexametaphosphate and rotated on a spinning wheel for 2h. Samples were then centrifuged at 18*g* for 1min at 10°C, the supernatants were pooled two-by-two and centrifuged at 2824*g* for 20min at 10°C. The microbe-containing pellet was resuspended in 10mL of 0,8% NaCl and samples were again pooled by two. The resuspended solution was then transferred onto 10mL of sterile Nycodenz and was centrifuged at 3220*g* for 40min. The bacterial layer was collected and a volume of 0,8% NaCl sufficient for a total of 35mL was added. This solution was centrifuged at 3220*g* for 15min at 10°C. Finally, the pellet was resuspended in 1mL of NaCl 0.8% to

wash the residual Nycodenz away and centrifuged at 1700*g* for 5min at 10°C. The final pellet was resuspended in 100µL TE buffer and kept at -20°C. RNA was extracted from this bacterial solution using a modified Griffiths' protocole, as follows: the 100µL were transferred into a MP Biomedicals™ Lysing Matrix E tube with 250µL of warmed 10% CTAB (Cetyl Trimethyl Ammonium Bromide) - 0,7M NaCl, followed by 250µL of 240mM K_2HPO_4/KH_2PO_4 (pH 8.0) and 500µL of phenol-chloroform-isoamyl alcohol (25:24:1) (pH 8.0). Samples were then bead-beaten using TissueLyser II (QIAgen) at 30Hz for 3min. The tubes were centrifuged at 16000*g* for 10min at 4°C and the supernatant was transferred into a new tube. One volume of chloroform-isoamyl alcohol (24:1) was added to the supernatant and centrifuged again at 16000*g* for 10min at 4°C. The supernatant was collected and 2 volumes of 30% PEG 6000-1,6M NaCl was added, and the tube was inverted to mix and was left at 4°C for at least 2h. The tubes were then centrifuged at 18000*g* for 30min at 4°C to precipitate RNA. The RNA pellet was then washed with 500µL of 70% ethanol and centrifuged at 18000*g* for 10min at 4°C. The pellet was then dried on the bench and resuspended in 50µL of RNAse-free water. RNA quality and quantity was verified using a 1% agarose gel and Nanodrop, and then sent for sequencing on an Illumina NovaSeq 6000 using paired-end 100bp with size-selected library. From the rhizosphere, 8 bacterial samples were isolated and, from the unplanted soils, 6 samples. Sequencing results were analysed as stated above with the rhizospheric miRNAs.
To confirm that miRNAs found in the rhizospheric bacteria originated from the plant and not the bacteria, miRNA sequences were searched for on the + and - strands of 3837 bacterial genomes[22], of which 1160 bacteria were isolated from plants. These miRNA sequences were also searched for in the genome of *Arabidopsis thaliana* TAIR10.1 in which they were confirmed at their corresponding position.
DESeq2 was used to identify differentially abundant miRNAs between bacteria isolated from the rhizosphere and bacteria isolated from unplanted soil, as described above. A second method, using edgeR, resulted in the same conclusions as with DESeq2, confirming the validity of the method.

Mutant Arabidopsis description

Five *Arabidopsis thaliana* mutants were grown in a mix of soil and sand (2:1) for a month, in individual pots. Four independent plants were used per condition. Mutants were provided by Hervé Vaucheret and Taline Elmayan. *RTL1* mutant over-expresses RTL1 protein which results in a suppression of siRNA pathway without affecting miRNAs[27]. *RTL1myc* over-expresses RTL1 protein flagged with Myc epitope, rendering RTL1 less active, so siRNA pathway is less suppressed than with *RTL1* mutant. *Ago1-27* mutant has AGO protein function partially impaired and is completely post-transcription gene silencing (PTGS) deficient [28,29]. *Dcl1-2* mutant has total loss of function of DCL1 protein resulting in low levels of miRNA and developmental problems[30]. *Hen1-4* mutant is miRNA defective but is also affected in some siRNA - PTGS[29]. HEN1 methylates siRNA and miRNA to maintain their levels and size, but also to protect them from uridylation and subsequent degradation[31].

DNA extraction, sequencing and qPCR

Arabidopsis thaliana mutants were grown for a month and sampled as follow: the soil easily detached from the root system was referred to as "distant rhizosphere" and the root, rhizoplane and close rhizosphere communities were named "root and close

rhizosphere" samples. DNA was extracted from the distant rhizosphere using a modified Griffiths' protocole that co-extracts DNA and RNA (as described above) and from the root and close rhizosphere using NucleoSpin Plant II kit (Macherey-Nagel). DNA was sent for 16S rRNA and ITS sequencing on an Illumina MiSeq in pair-ended mode (2x250) at the Centre d'expertise et de services Génome Québec (Montreal, Canada). In parallel, 16S rRNA gene (341F: 5'- CCTACGGGNGGCWGCAG- 3' and 534R: 5'- ATTACCGCGGCTGCTGGCA – 3') and ITS (ITS9F: 5'- GAACGCAGCRAAIIGYGA - 3' and ITS4R: 5'- TCCTCCGCTTATTGATATGC – 3') were quantified by qPCR, using the same primers as for sequencing. It should be noted that amplification of the ITS2 region with these primers (ITS9/ITS4) does not exclude plant ITS[32]. To counteract this, a ratio of fungal Amplicon Sequence Variants (ASVs) over non-fungal ASVs, found in each sample, was calculated and applied to the number of ITS copies quantified by qPCR. Within each well was 0,1µL of each primer (10µM), 4µL LightCycler® 480 SYBR Green I Master (final volume 6µL PCR mix) and 2µL of DNA (25ng/µL). Quantification of distant rhizosphere samples were performed with additional 0,67mg/mL BSA. 16S gene was quantified with the following program in 40 cycles: pre-incubation (95°C- 4min) / amplification (95°C – 30s ; 49°C – 1min ; 72°C – 1min) / 72°C-10min/ melting curve (95°C – 5s ; 49°C – 1min ; 97°C – continuous 5 measures/ °C) / cooling (40°C – 30s). Whereas ITS was quantified with 34 cycles of the program: pre-incubation (95°C- 5min) / amplification (95°C – 15s ; 60°C – 30s ; 72°C – 30s; 80°C – 5s) / melting curve (65°C à 95°C – 0.5°C/5s, continuous) / cooling (40°C – 30s). At the last step of amplification, a single measurement was performed and then continuously during the melting curve step. A minimum of 3 technical replicates was quantified. A number of copies of 16S or ITS was determined in comparison with a standard curve using serial dilutions of plasmids with cloned fragments (R^2=0,994 and R^2=0,9987 for root and close rhizosphere samples 16S and ITS, respectively, and R^2=0,998 and R^2=0,9882 for distant rhizosphere samples 16S and ITS).

Amplicon sequences processing

Amplicon sequencing data was processed with AmpliconTagger[33]. Briefly, contaminants and unpaired reads were removed, and remaining sequences were trimmed to remove adaptors and primers. Reads were then filtered for quality control such that reads having at least one N or having average phred score quality less than 20 were left out. For 16S analysis, a total of 3,776,292 reads passed the quality control and 1,230,944 sequences were successfully clustered in ASVs (DADA2 - PMID:27214047). For ITS analysis, 3,547,108 reads passed quality control and 824,610 sequences made it into ASVs. For both 16S and ITS data types, ASVs were filtered for chimaeras using DADA2's internal removeBimeraDeNovo (method = 'consensus') workflow followed by VSEARCH's UCHIME *de novo* (PMID:27781170). Each remaining ASV was assigned a taxonomic lineage by using the RDP classifier with the SILVA R138 database (PMID:23193283). ASVs assigned to Bacterial/Archaeal and Fungal microorganisms were kept for 16S and ITS data types, respectively and were rendered into ASV abundance tables that were used for downstream analyses.

Microbial communities' analyses

Analyses of ASVs composing the microbial communities were performed using the R package "phyloseq" v 1.32.0[34]. After checking the rarefaction curves (**Figure S4**), all samples seemed sufficiently sequenced. However, to study alpha-diversity, it is recommended to rarefy data. All reads were rarefied to the lowest number of reads found in a sample. To assess the diversity within samples, the number of observed ASVs, Shannon's and Simpson's indices were calculated. Significance of differences in alpha-diversity between mutants was tested using a linear model, whilst checking the normal distribution of residuals and using wild-type (WT) samples as a reference. If residuals did not follow a normal distribution, a general linear model (GLM) with gamma distribution and log scale was used.
The effect of miRNA/siRNA mutation in *Arabidopsis* on the structure of microbial communities was determined on non-rarefied data, using ordinations of Bray-Curtis distances (based on ASV abundance, ordination not shown) and Weighted UniFrac distances (abundance and phylogeny). Interpretation of 2 main axes was considered meaningful by using the broken-stick model (vegan package - rda and screeplot functions). The Adonis function (Permutational Multivariate Analysis of Variance Using Distance Matrices) was used to determine the statistical impact of miRNA/siRNA mutations on the microbiota structure, alongside checking group dispersion (betadisper function) and pairwise tests (pairwise.adonis function).

MiRNA sequences processing
Raw reads were trimmed for quality using Trimmomatic v0.39 (PMID:24695404) to remove the sequencing adapters with the Illumina clip setting parameter set to :5:10:4. and to remove the 5' and 3' flanking sequences containing bases having quality score below 3 and 30, respectively. Common contaminants (Illumina adapters, PhiX spike-in sequence) were removed with bbduk (from bbmap v38.11) ("BBMap" n.d.) with key parameters k=20 and minkmerhits=1. Remaining reads were filtered by size (nrc tools v1.3.2) such that reads having a length between 18 and 27 nucleotides were selected. Selected reads were mapped on the Arabidopsis (TAIR10/GCA_000001735.1) reference database (i.e., these genome versions correspond to the same genome versions as used in mirBase (PMID:16381832)) using BWA aln (PMID:19451168) with mismatch=1 and seed=5 parameters. Number of reads to align inside miRNA coordinates according to the corresponding genome model (i.e., gff) file were determined with bedtools v.2.29.2 (PMID:20110278) with the -F 0.95 argument meaning that at least 95% of each read has to be inside the coordinates of miRNAs (from the gff file) to be considered a valid hit. All remaining reads that mapped to these reference databases were selected for downstream miRNA abundance estimation across all samples. Remaining reads were normalised with RPKMs using edgeR v3.28.1 (PMID:19910308) with R v3.6.3 (R Core Team 2021)

In silico analysis of miRNA targets

To predict potential plant miRNA targets in rhizospheric bacteria, a workflow inspired from psRNAtarget([35]; PMID:21622958; 19802588), which is used to find miRNA targets in plant genomes, was implemented (https://github.com/jtremblay/MiRNATarget). This tool is based on specific pairing patterns between plant miRNA and target[36] and implements SSEARCH36 (from the fasta36 v36.3.8[37]package) which uses Smith-Waterman local alignment algorithm, resulting in the most optimal alignments. Multiple targets for each miRNA can be

identified. Based on the psRNAtarget rules for miRNA-target recognition, an *e*-value was calculated for each alignment and only those with an *e*-value ≤ 5 were retained. The position of the targeted region was noted with respect to the neighbouring coding DNA sequences (CDS): the target sequence could be inside a CDS, at the 5' or 3' untranslated region (UTR) or in other UTRs, intercistronic regions, overlapping CDSs or elsewhere, further away from any CDS. If the target sequence was in the latter category, *i.e.,* too far from any CDS, it was discarded from the analysis, because it was considered that a miRNA in that area would not affect mRNA translation. For functional analysis, the closest CDS to the miRNA target region was investigated. The CDSs of 3837 bacterial genomes[22] were predicted using Prodigal v2.6.3[38]. Cellular function of these CDSs were inferred by alignment with ortholog genes referenced in the KEGG database (PMID:10592173; 31441146), identified with DIAMOND blastp v2.0.8[39]. Only results with an *e*-value < $1e^{-10}$ were kept for downstream analyses. For taxonomic analyses, the number of targets in a certain family was normalised by the abundance of this family in the whole dataset.

Discussion

Taken together, the results presented here suggest that a hitherto undetected mechanism can contribute to shaping the rhizosphere microbiome. We showed that plants secrete specific miRNAs in their rhizosphere, which are then taken up by bacteria. These miRNAs appear to target bacterial genes mainly related to the uptake and metabolism of amino acid, a key nutrient in the rhizosphere. Accordingly, plants mutated in small RNA pathways harboured significantly different microbial communities in their rhizosphere.

We detected plant miRNAs in the rhizospheres of two phylogenetically distant plants, *Arabidopsis thaliana* and *Brachypodium distachyon*. In plants, miRNAs are well known to intervene in development, in response to abiotic and biotic stresses, and during symbiotic or parasitic processes[40]. Whilst miRNAs have been investigated in most plant tissues and in response to various biotic and abiotic conditions, such as microbial infections, drought or salinity, this is the first time the rhizosphere has been scanned for their presence. In comparison with the diversity of miRNAs found in plant tissues, only a select few were found in abundance in the rhizosphere, which may indicate a selection process *in planta*. Interestingly, in roots, miRNAs have specific expression patterns, some being exclusively produced in certain cell types or root zones[12]. By comparing the major miRNAs found in the rhizosphere of *Arabidopsis* with those previously found in roots[12], all of them were detected in roots but these rhizospheric miRNAs did not seem to be specifically localised in a certain cell type. A majority of our rhizospheric miRNAs, including the two most abundant, ath-miR158a and ath-miR161.1, were found to be highly enriched in the early meristematic zone[12]. Many root exudates, such as exDNA, soluble compounds and mucilage, are produced and secreted, by border cells, in this region of the root tip[41]. Thus, we can hypothesise that these rhizospheric miRNAs are likely secreted by roots in the early meristematic zone, at the root tip. Another interesting finding, in both *Arabidopsis* and *Brachypodium,* is the identity of these rhizospheric miRNAs : many are part of the miR159 family, which is extensively studied, due to its importance in numerous spheres of plant life. Members of the miR159 family are highly conserved throughout the plant kingdom and target a family of genes encoding MYB transcription factors, regulating plant

development, flowering and response to abiotic and biotic stresses[42]. The fact that such phylogenetically distant species have a set of common rhizospheric miRNAs, some of which being essential to many aspects of plant biology, suggests that the secretion of these miRNAs may be a conserved trait across plants.

Given our increasing knowledge about small RNAs' function in plant-microbe interactions, it seems relevant that root-secreted miRNAs may equally be involved. Interactions *via* small RNAs, including miRNAs, with fungal partners have been covered by previous studies[14–16,18,43], but miRNA-based interplay with bacterial partners has been neglected. By sequencing the RNA content of bacteria isolated from the rhizosphere, we identified 10 ath-miRNAs, which were absent from bacteria isolated from unplanted soils, suggesting that bacteria are capable of uptaking surrounding miRNAs, in the rhizosphere. The incorporation of eukaryote miRNAs in bacteria is consistent with their ability to absorb environmental nucleic acids, such as extracellular DNA, through natural competence. Competent bacteria use their type IV pili to bind and pull extracellular double-stranded DNA through their membrane or peptidoglycan layer[44]. Although not all bacteria have natural competence, most rhizospheric bacteria do encode type IV pili in their genome (Yergeau, *et al.* unpublished). In the same way that some bacteria secrete small RNAs in outer membrane vesicles[45], it is proposed that bacteria may internalise external DNA *via* vesiduction[46], *i.e.* membrane fusion of a vesicle containing DNA or RNA. In the case of transporting miRNAs, many questions remain unanswered: In vesicles, are miRNAs conveyed on their own or are they bound to argonaute proteins, as in the RNA-Induced Silencing Complex (RISC)? Are miRNAs secreted in the rhizosphere as such and internalised by naturally competent bacteria? These intriguing questions were outside the scope of the current study.

To explore the impact of plant miRNAs on the rhizospheric microbiota, we examined the microbial communities' composition of *Arabidopsis* mutants affected in the biosynthesis of miRNA and/or siRNA. Based on 16S rRNA and ITS amplicon sequencing, we observed disrupted microbial communities in roots and rhizosphere of mutants compared with wild-type plants, suggesting an important role of small RNAs in constituting microbial communities. For instance, microbial communities in the roots and rhizosphere of *ago1-27* and *RTL1myc* mutants resembled further those of an unplanted soil than those of wild-type (WT) samples. This suggests these mutations lead to a certain dysbiosis in the roots and rhizosphere microbiota. Bacterial communities from mutants *dcl1-2* and *RTL1*, impaired in either miRNA or siRNA pathways, were the most similar to WT which could reveal some compensatory mechanism when one type of small RNA is missing, to maintain their regulatory role. Previous research has shown that in absence of siRNA, miRNA levels are increased, because they compete for HEN1 methylation[47]. On the contrary, fungal communities from these two mutants were the most distinct from WT, with increased proportions of Chytrids, notably from the Olpidium genus. Amongst all mutations, the *hen1-4* mutation least affected fungal communities, which could be explained by the fact that some miRNA families are not impacted by the lack of methylation[48] and could be maintained in these plants.
Bacterial and fungal diversities were significantly higher in mutated plants, in both roots and close rhizosphere and in distant rhizosphere, in comparison with WT communities. Microbial diversity varies in inverse proportion to plant selection, for example, microbial diversity diminishes from bulk soil to the rhizosphere, and finally,

to the endosphere, as the selection strength increases[49]. A high diversity in mutants could reflect a weaker selection from the plant, which may be directly or indirectly orchestrated by plant miRNAs and/or siRNAs. As demonstrated in a previous study on the gut microbiota[20], plant miRNAs may have an indirect effect on the microbiota, by impacting the secretion of bacterial metabolites, which, in turn, modify the growth of surrounding microbes. Anyhow, the microbial data from these mutants have to be interpreted with caution due to the low sampling size and the impact of the mutation on the plant's physiology, as a whole.

Whilst mechanisms behind miRNA-based interference in plants seem to be consensual, the way eukaryotic miRNAs target and repress bacterial genes must be determined. Plant miRNAs inhibit target gene expression *via* mRNA cleavage or translation repression, through near perfect sequence complementarity[50]. Hypotheses from previous research assumed that eukaryotic miRNAs target bacterial genes in the same manner, through sequence complementarity, and were somewhat confirmed through *in vitro* and *in vivo* experiments[19,20]. For this study, the search of plant miRNA targets in bacterial genomes was automated using the established rules for plant miRNA targeting, used in *psRNAtarget*. Our predictions suggest that the 10 ath-miRNAs internalised in rhizobacteria can target many genes, in both *Arabidopsis* rhizospheric bacteria and non-plant-associated bacteria. Although this may come as a surprise, previous research has established that plant miRNAs can target and repress bacterial genes in the gut microbiota[20]. The most interesting finding was that these plant miRNAs target preferentially bacterial genes related to amino acid transport and metabolism in typical plant-associated bacterial taxa. Nitrogen being the most limiting factor for plant growth and amino acids being one of the most rapidly cycled nitrogen sources in soils[51], it would make sense for plant miRNAs to indeed repress the expression of such genes in bacteria, to increase their competitiveness to absorb this organic N source. More specifically, the targeted microbial genes code for amino acid transporters and enzymes related to nitrogen metabolism. For instance, the 10 ath-miRNAs reported here and found in rhizobacteria, target genes encoding a serine hydroxymethyltransferase, an amino acid ABC transporter substrate-binding protein, a 3-isopropylmalate dehydratase large subunit or an ABC transporter permease (data not shown). It is important to bear in mind that these genes are *in silico* predicted targets and will need to be confirmed *in vivo*. If these predicted targets are confirmed, it would indicate that the rules governing plant miRNA - bacterial mRNA pairing are the same as those for plant miRNA - mRNA, as described in *psRNAtarget*.

There are still many unanswered questions concerning the function of rhizospheric plant miRNAs and their impact on the rhizomicrobiome. Future work is required to validate the predicted bacterial targets and to establish the effect of rhizospheric miRNAs on the composition and especially the activity of the rhizospheric microbiota. The use of miRNA-encoded peptides (miPEPs)[52] is an excellent way to investigate miRNA-related mechanisms in plants, combined with single miRNA mutants to establish the role of specific miRNAs in controlling the rhizomicrobiota. Nevertheless, for the first time, we detected plant miRNAs in the rhizosphere and showed that they were also found inside rhizospheric bacterial cells, potentially targeting bacterial genes related to amino acid metabolism and transportation. Our study suggests a completely novel way that plants can use to shape their rhizospheric microbiota, challenging the current paradigm of rhizosphere microbial assembly.

Beyond providing exciting new insights into the growing area of research that are plant-microbe interactions through small RNAs, this study highlights that miRNA may be a potential tool for microbiota engineering. Indeed, to render our cultures more resilient to an increasingly stressful environment, many methods are used to optimise the plant microbiome, of which miRNAs may be a part of, in the future.

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Figures

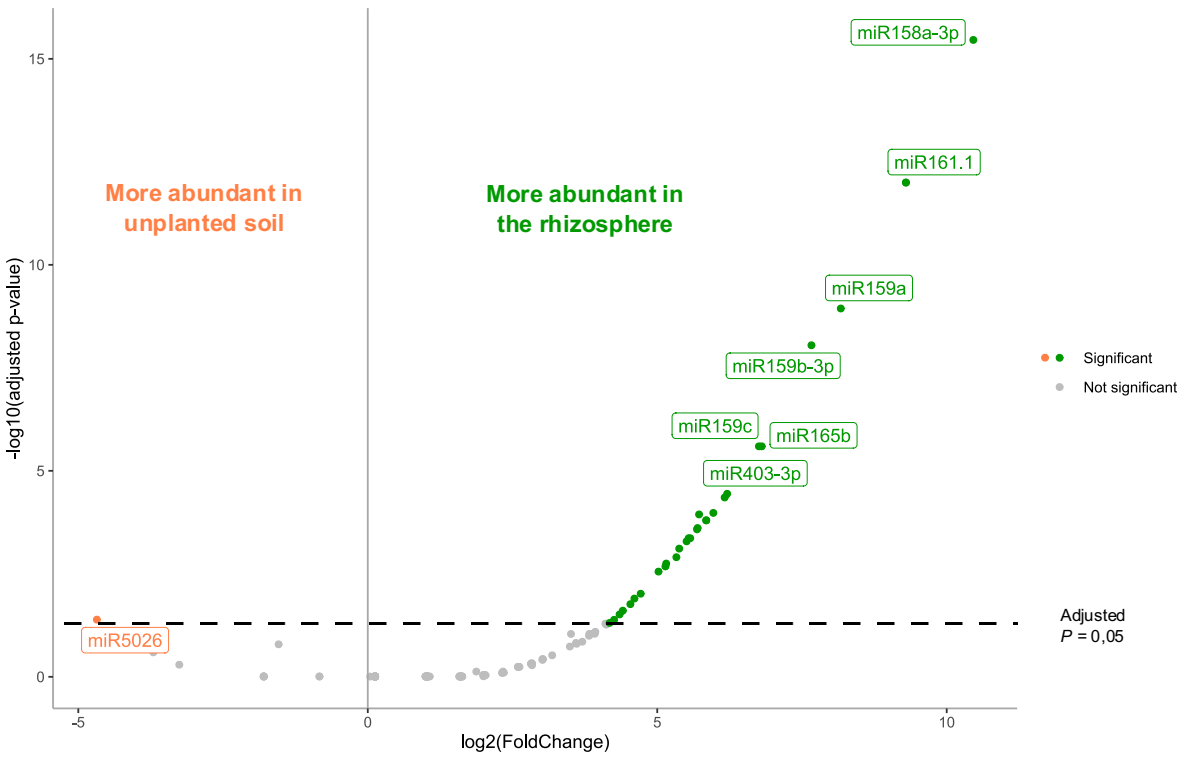

Figure 1: *Arabidopsis thaliana* miRNAs identified in the rhizosphere. Differentially abundant miRNAs found in the rhizosphere of *A. thaliana* and unplanted soil, resulting from DESeq2 analysis. 29 miRNAs were significantly more abundant in the rhizosphere than in unplanted soil (points on the right). *P*-values were adjusted using the Benjamini & Hochberg method.

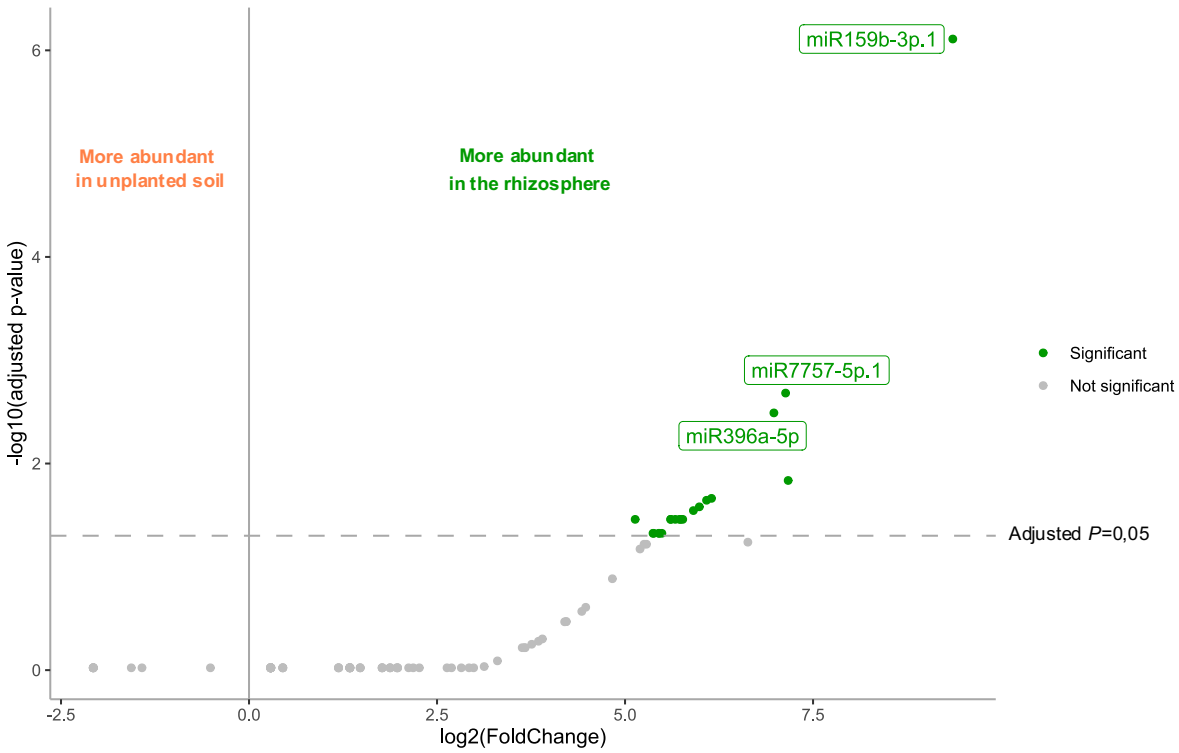

Figure 2: *Brachypodium distachyon* miRNAs identified in the rhizosphere. Differentially abundant miRNAs found in the rhizosphere of *B. distachyon* and unplanted soil, resulting from DESeq2 analysis. 20 miRNAs were significantly more abundant in the rhizosphere than in unplanted soil (points on the right). *P*-values were adjusted using the Benjamini & Hochberg method.

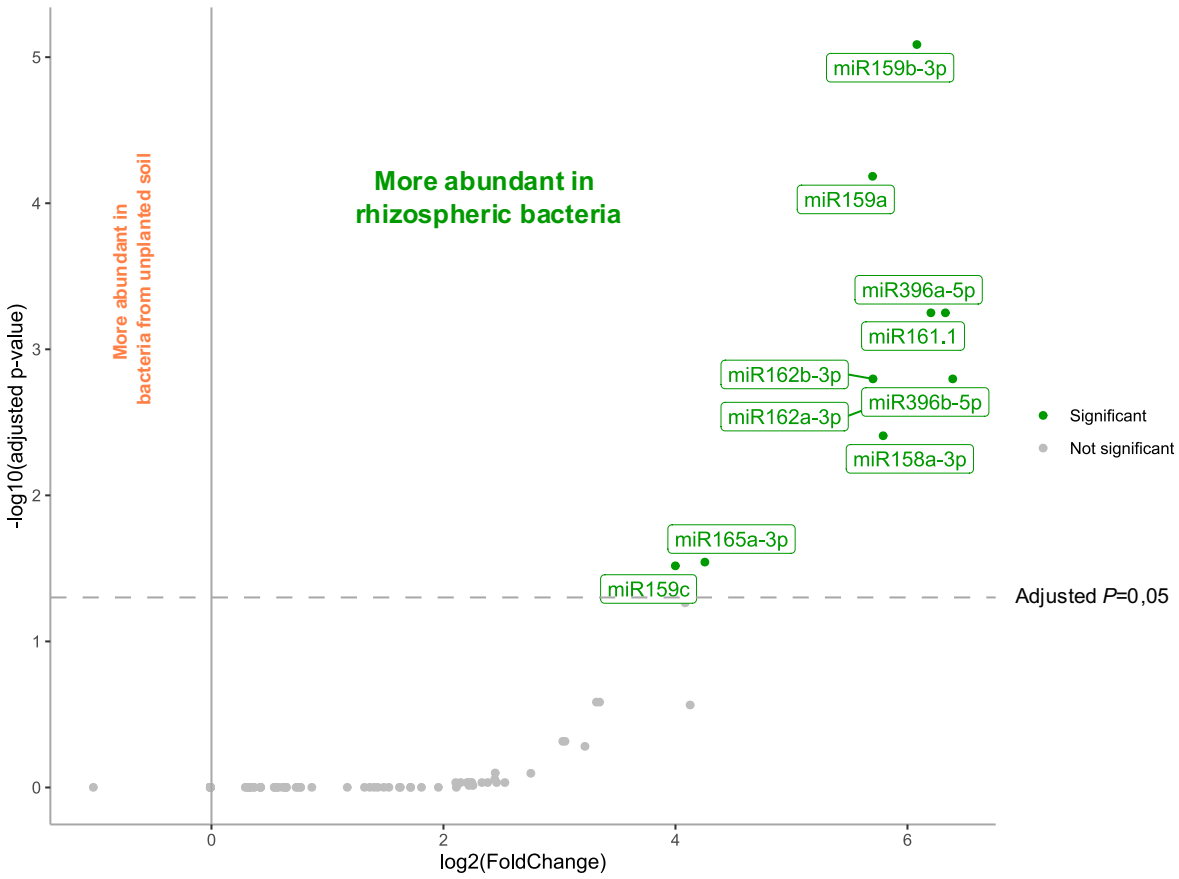

Figure 3: *Arabidopsis thaliana* miRNAs identified in bacteria isolated from rhizospheric and unplanted soil samples. Differentially abundant miRNAs found in bacteria from the rhizosphere and unplanted soil, resulting from DESeq2 analysis. 10 miRNAs were significantly more abundant in the rhizospheric bacteria than in those isolated from unplanted soils (points on the right). *P*-values were adjusted using the Benjamini & Hochberg method.

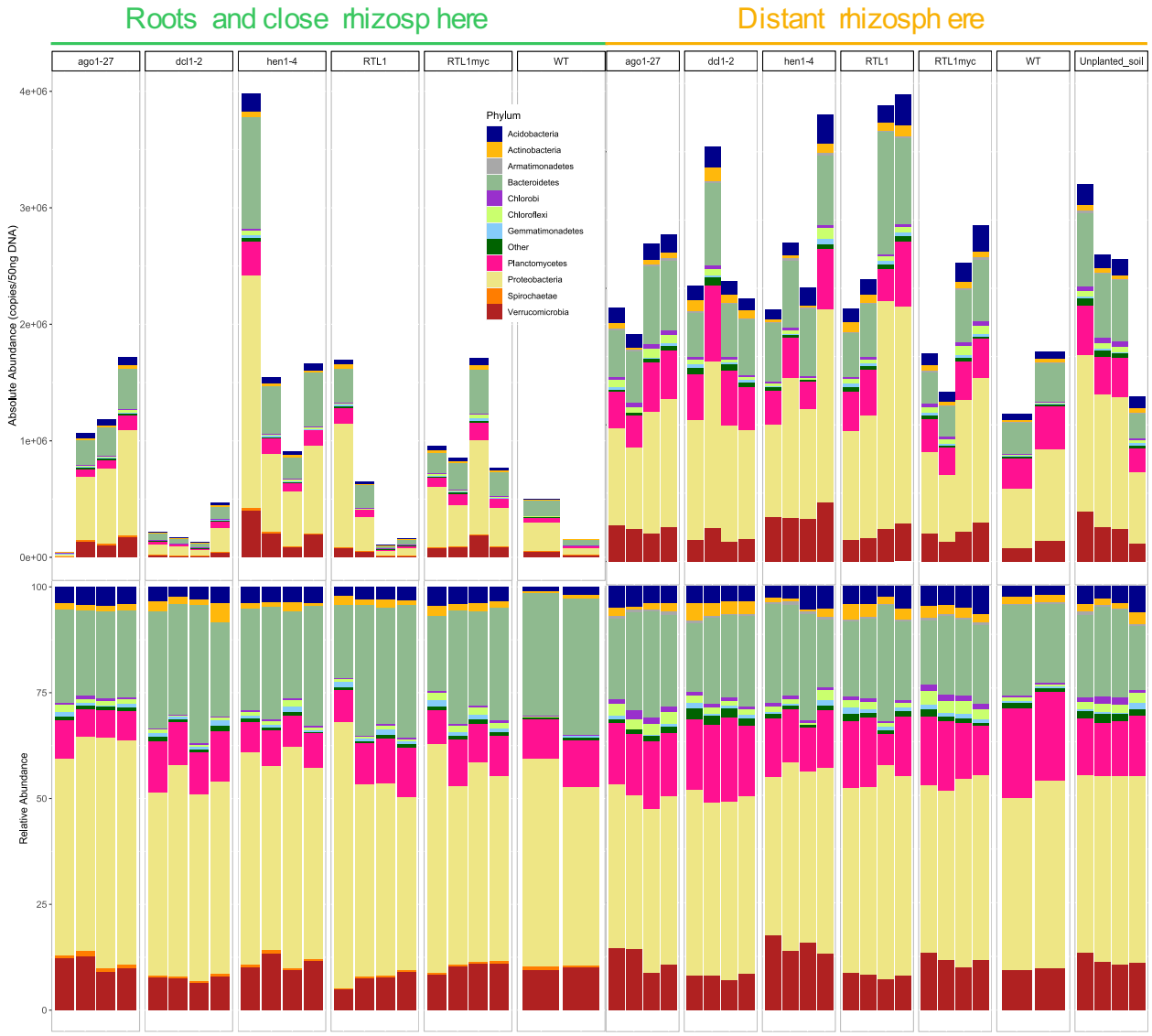

Figure 4: Taxonomic profiles of bacterial communities in the roots and rhizosphere of mutant and wild-type *Arabidopsis thaliana*. Absolute and relative abundance of 10 major bacterial phyla present across samples.

Figure 5: Taxonomic profiles of fungal communities in the roots and rhizosphere of mutant and wild-type *Arabidopsis thaliana*. Absolute and relative abundance of fungal phyla in samples.

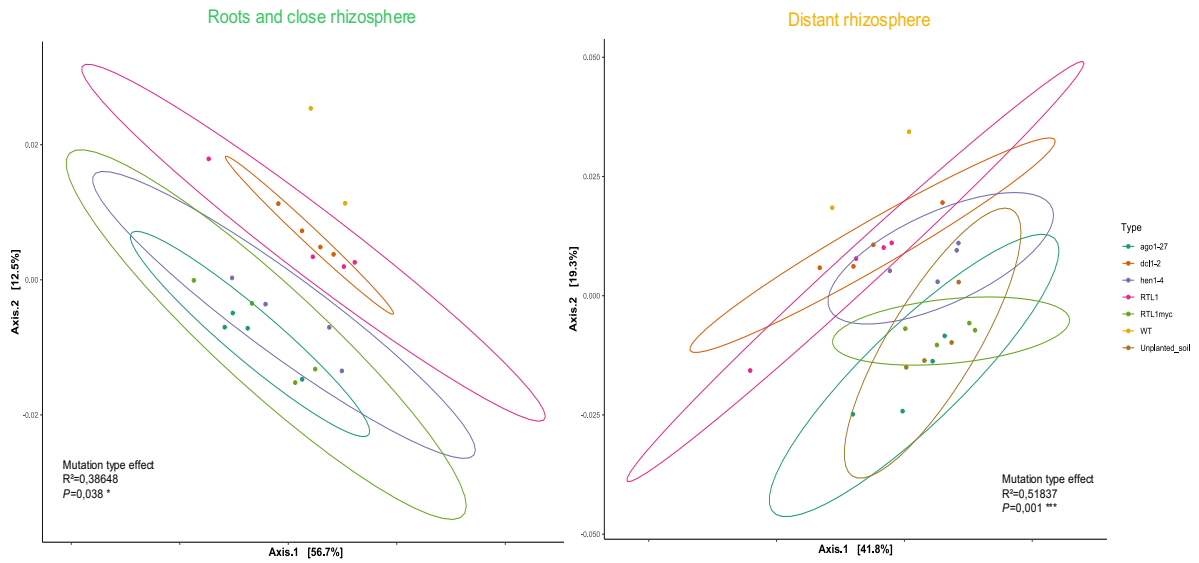

Figure 6: Principal Coordinate Analysis (PCoA) ordination, using Weighted UniFrac distances, of root and rhizospheric bacterial communities. Statistical analysis performed using a PERMANOVA test with distance matrices (adonis function) : *P*<0,05 *, *P*<0,01 **, *P*<0,001 ***.

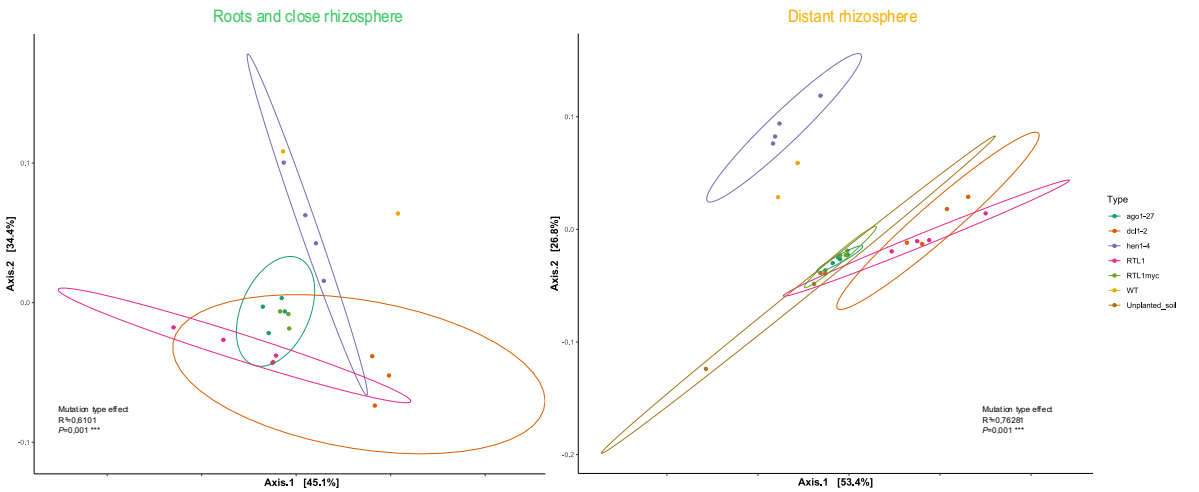

Figure 7: Principal Coordinate Analysis (PCoA) ordination, using Weighted UniFrac distances, of root and rhizospheric fungal communities. Statistical analysis performed using a PERMANOVA test with distance matrices (adonis function) : *P*<0,05 *, *P*<0,01 **, *P*<0,001 ***.

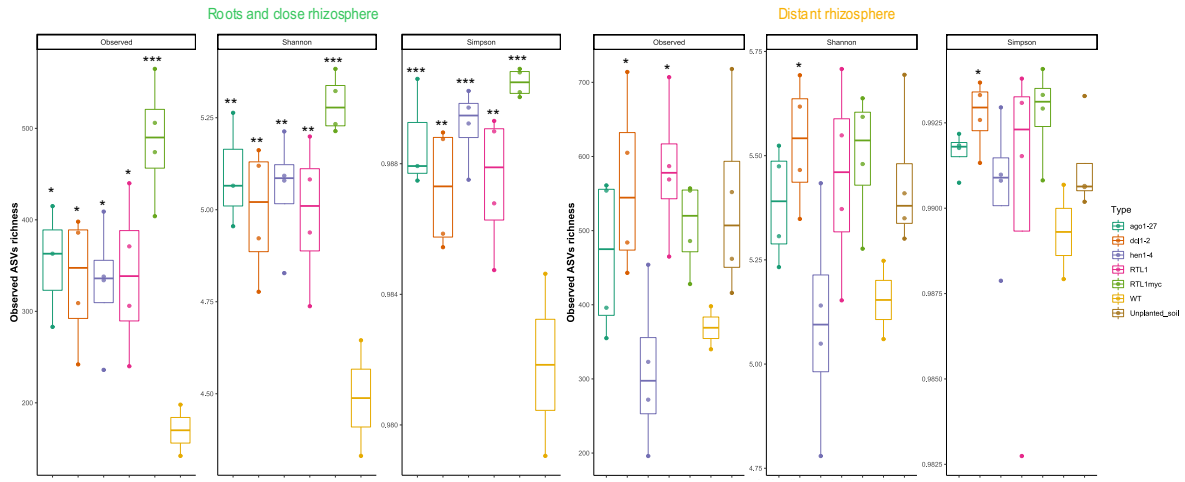

Figure 8: Alpha diversity of root and rhizospheric bacterial communities: observed number ASVs, Shannon's index and Simpson's index. Statistical analysis was performed using a linear model (*P*<0,05 *, *P*<0,01 **, *P*<0,001 ***).

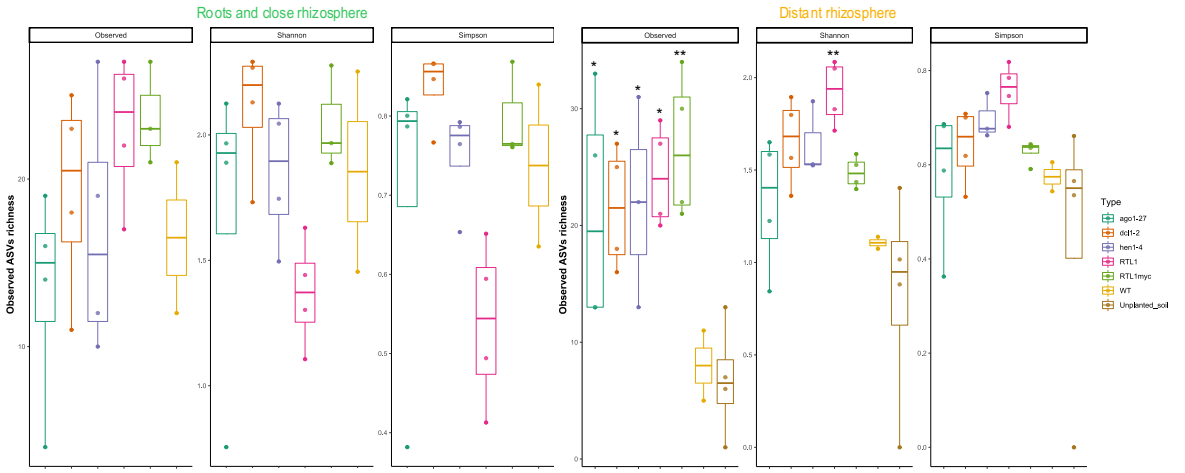

Figure 9: Alpha diversity of root and rhizospheric fungal communities: observed number ASVs, Shannon's index and Simpson's index. Statistical analysis was performed using a linear model (*P*<0,05 *, *P*<0,01 **).

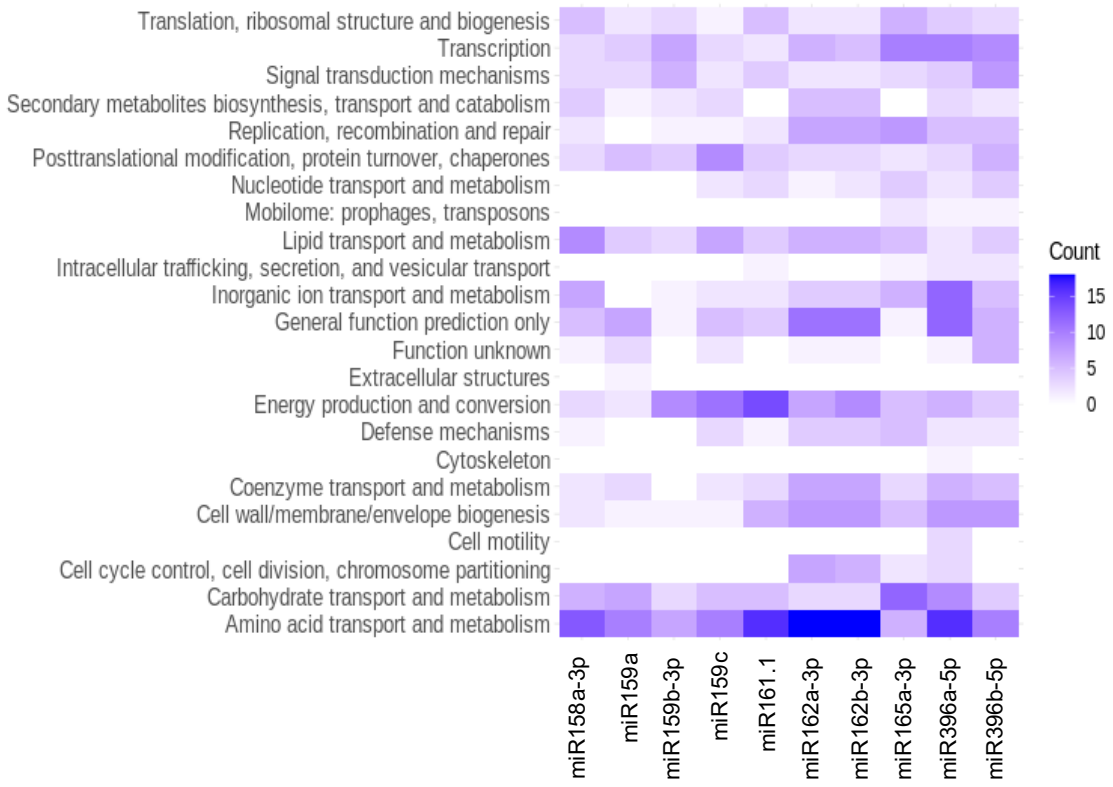

Figure 10: Predicted functional targets, in *Arabidopsis* rhizospheric bacteria, of the 10 ath-miRNAs found in rhizobacteria.