

Plant miRNAs in the rhizosphere target microbial genes

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Abstract

Background 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-associated microbial communities.

Results To explore these questions, we performed small RNA sequencing in search of miRNAs in the rhizosphere of two 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. Additionally, confronting synthetic rhizospheric miRNAs with bacterial cultures resulted in modulation of gene expression, suggesting a functional role of plant miRNAs in regulating microbial activity, in the rhizosphere.

Conclusions 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 stresses¹. 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 engineering². 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}. These same factors are involved in the establishment and functioning of the rhizospheric microbiota, however, the plant component is consistently emphasised in the search for microbial engineering techniques. 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 it is also heavily dependent on the biogeography of soil microorganisms³.

Roots exudates are of diverse nature such as sugars, peptides, amino acids, nucleic acids, nucleotides, fatty acids or secondary metabolites⁸. 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 rhizodeposits⁹. This exDNA forms a matrix that functions as a trap surrounding the root mucilage to attract and immobilise pathogens¹⁰, somewhat shaping the microbiota. Likewise, we hypothesised that small RNAs, notably microRNAs (miRNAs), are exuded through the roots, forming a supplementary class of rhizodeposits. These regulatory ~21nt non-coding RNAs are found in *Arabidopsis* roots and over half of them are expressed in a compartment-specific manner¹¹. Interestingly, whilst no previous studies provided evidence of miRNA secretion in the rhizosphere, a large number of miRNAs are enriched at the root tip, in the early meristematic zone¹¹. 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 power¹², potentially through root-secreted miRNAs.

Throughout the last decade, 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 genes¹³ and *Arabidopsis thaliana* delivered small RNAs *via* vesicles to the attacking *Botrytis cinerea*, inhibiting virulence¹⁴. 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 gene in wheat¹⁵. These small RNAs mediate cross-kingdom communication and are often the result of the famous arms' race between plants and fungal pathogens¹⁶.

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 microbiota¹⁷. 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 expression¹⁸. Regarding the role of miRNAs in the gut microbiota and the commonalities between the intestine and the rhizosphere¹⁹, it seems reasonable that root-secreted miRNAs might be one of the important factors that shape the biodiversity and activity of this interacting rhizomicrobiome²⁰. 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 present an initial study that leveraged small RNA sequencing to characterise the relationship between *Arabidopsis thaliana* and *Brachypodium distachyon*'s rhizosphere and their respective microbiota. This led us to further explore their function in regard to surrounding microorganisms, through microbial community analyses of small RNA - lacking plants and transcriptomics of bacteria confronted to synthetic miRNAs. With these results, we demonstrate that plants may use miRNAs, in their rhizosphere, to modulate the composition and/or activity of surrounding microbes. A greater understanding of the interactions at play in the plant holobiont will, in turn, advance our abilities to grow plants in coherence with their microbial environment.

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 100 µL, 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 Trimmomatic²¹ (v0.39) to remove the sequencing adapters with the following Illumina clip settings :5:10:4 and to remove the 5' and 3' flanking sequences containing bases having quality scores below 3 and 30, respectively. Common contaminants, such as Illumina adapters and PhiX spike-in sequences, were removed using BBDuk (BBMap v38.11, <https://github.com/BioInfoTools/BBMap>), with key parameters: k=20 and minkmerhits=1. Remaining sequences were then 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 against either *A. thaliana* (TAIR10/GCA_000001735.1) or *B. distachyon* (bd21/GCF_000005505.1) reference genomes, which are the same as those used in miRBase²², using BWA-aln²³ with the following parameters: mismatch=1 and seed=5. Using BEDTools v.2.29.2²⁴ 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.

A differential analysis was performed using DESeq2²⁵ to compare the abundances of each miRNA between two conditions (unplanted soil VS rhizosphere). The code and data for this analysis are available on GitHub. For each miRNA, raw counts were 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 was considered as a normalisation factor or size factor. Normalised counts were 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 two-sided 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 5 g of rhizospheric soil, using a density gradient. Samples were added to 30 mL of 0.2% sodium hexametaphosphate and rotated on a spinning wheel for 2 h. Samples were then centrifuged at 18 *g* for 1 min at 10°C, the supernatants were pooled two-by-two and centrifuged at 2824 *g* for 20 min at 10°C. The microbe-containing pellet was resuspended in 10 mL 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 40 min. 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 15 min at 10°C. Finally, the pellet was resuspended in 1 mL of NaCl 0.8% to wash the residual Nycodenz away and centrifuged at 1700 *g* for 5 min at 10°C. The final pellet was resuspended in 100 µL TE buffer and kept at -20°C. RNA was extracted from this bacterial solution using a modified Griffiths' protocol, as follows: the 100 µL were transferred into a MP Biomedicals™ Lysing Matrix E tube with 250 µL of warmed 10% CTAB (Cetyl Trimethyl Ammonium Bromide) - 0.7 M NaCl, followed by 250 µL of 240 mM K₂HPO₄/KH₂PO₄ (pH 8.0) and 500 µL of phenol-chloroform-isoamyl alcohol (25:24:1) (pH 8.0). Samples were then bead-beaten using TissueLyser II (QIAgen) at 30 Hz for 3 min. The tubes were centrifuged at 16000 *g* for 10 min 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 10 min at 4°C. The supernatant was collected and 2 volumes of 30% PEG 6000-1.6 M NaCl was added and the tube was inverted to mix and was left at 4°C for at least 2 h. The tubes were then centrifuged at 18000 *g* for 30 min at 4°C to precipitate RNA. The RNA pellet was then washed with 500 µL of 70% ethanol and centrifuged at 18000 *g* for 10 min at 4°C. The pellet was then air dried and resuspended in 50 µL 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 100 bp with size-selected library. Eight bacterial samples were processed from the rhizosphere and six were processed for unplanted soils. Sequencing results were processed 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 3,837 bacterial genomes²⁶, of which 1,160 bacteria were isolated from plants. These miRNA sequences were also searched for in the genome of *A. thaliana* TAIR10.1 in which they were confirmed at their corresponding position.

DESeq2 was used, as described above, 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, validating the method.

Mutant Arabidopsis thaliana description

Five *A. thaliana* mutants were grown in a mix of soil and sand (2:1) for a month, in individual pots. Mutants were provided by Hervé Vaucheret and Taline Elmayan (IJPB, INRAE, Versailles, France). *RTL 1* mutant over-expresses RTL1 protein which results in a suppression of siRNA pathway without affecting miRNAs²⁷. *RTL 1myc* over-expresses RTL1 protein flagged with Myc epitope, rendering RTL1 less active, so siRNA pathway is less suppressed than with *RTL 1* 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 problems³⁰. *Hen1-4* mutant is miRNA defective, but is also affected in some siRNA - PTGS²⁹. HEN1 methylates siRNA and miRNA to maintain their levels and size, but also to protect them from uridylation and subsequent degradation³¹.

DNA extraction, sequencing and qPCR

A. thaliana mutants ($n = 4$) and wild-type plants ($n = 2$) were grown for a month and sampled as follows: 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’ protocol 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 (341F: 5'- CCTACGGGNGGCWGCAG- 3' and 534R: 5'- ATTACCGCGGCTGCTGGCA – 3') and ITS (ITS9F: 5'- GAACGCAGCRAAIIIGYGA - 3' and ITS4R: 5'- TCCTCCGCTTATTGATATGC – 3') sequencing on an Illumina MiSeq in paired-end mode (2x250 bp) at the Centre d'expertise et de services Génome Québec (Montreal, Canada). In parallel, 16S rRNA gene and ITS 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 ITS³². 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 0.1 µL of each primer (10 µM), 4 µL LightCycler® 480 SYBR Green I Master (final volume 6 µL PCR mix) and 2 µL of DNA (25 ng.µL⁻¹). Quantification of distant rhizosphere samples were performed with additional 0,67 mg.mL⁻¹ BSA. 16S gene was quantified with the following program in 40 cycles: pre-incubation (95°C- 4 min) / amplification (95°C – 30 s ; 49°C – 1 min ; 72°C – 1 min) / 72°C-10 min/ melting curve (95°C – 5 s ; 49°C – 1 min ; 97°C – continuous 5 measures/ °C) / cooling (40°C – 30s). Whereas ITS was quantified with 34 cycles of the program: pre-incubation (95°C- 5 min) / amplification (95°C – 15 s ; 60°C – 30 s ; 72°C – 30 s ; 80°C – 5 s) / melting curve (65°C à 95°C – 0.5°C/5 s, continuous) / cooling (40°C – 30 s). 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 AmpliconTagger³³. 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 processed to generate ASVs (DADA2³⁴). 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's³⁵ UCHIME *de novo*. Each remaining ASV was assigned a taxonomic lineage by using the RDP classifier with the SILVA³⁶ R138 and UNITE (version: *sh_qiime_release_s_10.05.2021*) databases for 16S and ITS data types respectively. ASVs assigned to bacterial/archaeal and fungal microorganisms were kept and rendered into ASV abundance tables. These tables were transformed, using qPCR data measuring 16S and ITS copies in each sample, into "absolute" abundance tables of ASVs which were used for downstream analyses.

Microbial communities analyses

Analyses of ASVs composing the microbial communities were performed using the R package "phyloseq" v 1.32.0³⁷. The R code and data used to generate the figures related to microbial analyses are available on GitHub. After checking the rarefaction curves (**Extended Data Fig.1**), 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 (see our GitHub for more details on the rarefaction process). 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" or "link" scale were used. All tests performed were, by default, two-sided.

The effect of miRNA/siRNA mutation in *A. thaliana* on the structure of microbial communities was determined on the abundance table (*i.e.* abundance was however adjusted by 16S/ITS copy number determined by qPCR as described earlier in the methodology), using ordinations of Bray-Curtis distances (based on ASV abundance, ordination not shown) and Weighted UniFrac distances (abundance and phylogeny). These distances were represented within a Principal Coordinate Analysis (PCoA), where each sample's microbiota is illustrated by a point and the closer two points are, the more similar their microbiota is. Interpretation of two 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).

In silico analysis of miRNA targets

To predict potential plant miRNA targets in bacterial genomes, we implemented a workflow³⁸ named *mirnarget 1.0 : miRNA target finder* (available on GitHub), which was inspired from the plant miRNA target finder, *psRNAtarget*^{39–41}. This tool is based on specific pairing patterns between plant miRNA and target⁴² and implements SSEARCH36 (from the fasta36 v36.3.8⁴³ package) which uses the Smith-Waterman local alignment algorithm, resulting in 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.

To compare potential targets of plant miRNAs in different bacterial genomes, we created two groups of genomes: *A. thaliana* rhizospheric bacteria (ARA) and non plant-associated bacteria (NPA). We used genomes from 140 ARA and 169 NPA bacteria described in Levy, *et al.*, 2018. The NPA group was randomly re-sampled to retrieve only 140 bacteria, for a homogeneous comparison.

The position of the targeted regions were noted with respect to the neighbouring coding sequences (CDS) (**Suppl. Info. Fig. 1**) : the target sequence could be inside a CDS, at the 5' or 3' flanking regions (FLR) or in other short/very short inter-CDS regions, overlapping CDSs or elsewhere, further away from any CDS. Targets in the latter category were positioned at a distance superior to 100 nt from the 5' end or at a distance superior to 350nt from the 3' end of the CDS. These distances were chosen as they correspond to the longest UTRs that are implicated in gene regulation. Targets in this category were discarded from the analysis, because it was considered that a miRNA in that area would not affect mRNA translation. The annotation of targeted regions and extraction of the closest CDSs were carried out with our tool "align2cdsRegions", available on GitHub.

Sequences of targeted CDSs were retrieved using the *getfasta* tool from BEDTools²⁴ (v2.30.0) and GO (Gene Ontology) terms were attributed using InterProScan⁴⁴ (v5.59-91.0), on Galaxy⁴⁵. For each domain of GO (*i.e.* Biological Processes, Cellular Component, Molecular Function) and for each targeted CDS, a GO ancestor and their description were associated and a level of hierarchical ontology was attributed, as described in Manjang, *et al.*, 2020⁴⁶.

Bacterial growth and miRNA treatment

Variovorax paradoxus EPS was grown in 5% yeast extract (YE), at 30°C with 250 rpm shaking. *Bacillus mycoides* YL123 was grown in tryptic soy broth (TSB), at 25°C with 250 rpm shaking. Bacterial stocks were plated on YE or TSB and single colonies were selected for liquid culture overnight ($n = 3$), in 20mL YE or TSB. In the morning, cultures were normalised as such: the optical density (OD) of overnight cultures was measured at 600 nm, which were then centrifuged at 5000 rpm for 5 min to collect the bacterial pellet. The supernatant was removed and a calculated volume of medium was added to the pellet in order to reach OD = 1 for *B. mycoides* (*i.e.* $7,36.10^8$ CFU/mL) and OD = 0.3 for *V. paradoxus* (*i.e.* $9,99.10^8$ CFU/mL). Bacterial cultures were left to grow until the exponential phase was reached, which

was determined previously (4-5 h for both strains). OD was then measured to check that the maximum number of cells/mL for RNA extraction was not exceeded (10^9 cells/mL). At this point, 1 µg of the miRNA mix or scrambled mix was applied to the cultures and gently mixed (15 µL of each miRNA solution diluted at 10 µM). Cultures were normalised to reach the same number of bacterial cells per mL. The volume of the cultures was varied to mimic natural differences in rhizosphere volume. Total concentration of miRNAs ranged between 65 nM-104 nM for *B. mycooides* and 45 nM-85 nM for *V. paradoxus*. The same cultures were sampled after 20 min and 120 min of incubation, in order to evaluate the evolution of gene expression in a same biological sample in response to a short and long confrontation with miRNAs. The miRNA mix was composed of 6 rhizospheric miRNAs : miR159a, miR159b, miR159c, miR161.1, miR158a and miR165b. The scrambled mix was composed of 6 RNA sequences with the same nucleic acids as the respective miRNAs but in a random order. These sequences are detailed in **Suppl. Info. Table 1**. To resemble mature plant miRNAs found in the rhizosphere, these synthetic miRNAs were single-stranded with a 3' methyl group. They were synthesised by Integrated DNA Technologies (IDT).

RNA extraction for bacterial transcriptomics

After incubation, 1 mL of every culture was sampled and centrifuged at 5000 rpm for 5 min. The bacterial pellet was resuspended in 100 µL of a lysozyme solution : 1 mg.mL^{-1} for *V. paradoxus* (Gram -) and 3 mg.mL^{-1} for *B. mycooides* (Gram +). After vortexing the resuspension, the samples were left to incubate for 15 min at room temperature. The following steps used solutions from the RNeasy Plant Mini Kit (Qiagen). The guanidine-thiocyanate-based buffer RLT was added to the samples for further lysis (600 µL) and vortexed for 10 s. For *B. mycooides* samples, an additional step was necessary: the 700 µL were transferred to a sterile 2 mL Safe-Lock tube containing 100 mg glass beads (0.1 mm diameter) and 100 mg zirconia/silica beads (0.5 mm diameter). Beads were resuspended by pipetting then cells were disrupted using Fast Prep for 50 s (5 m/s), followed by 5 min on ice and another 50 s of Fast Prep. Tubes were centrifuged at $10\,000g$ for 1 min and supernatant was transferred to a new tube. The next steps were common to both strains. An equal volume of 70% ethanol was added to the lysed samples and mixed by pipetting. The lysate was transferred to an RNeasy spin column (pink column) in a 2 mL collection tube and centrifuged at 10 000 rpm for 30 s, then the flow-through was discarded. Columns were first washed with 700 µL of the RW1 wash buffer, followed by 500 µL RPE buffer and again with 500 µL RPE buffer. Collection tube was changed and the column was centrifuged for 1 min at maximum speed to dry the membrane off. To elute RNA, columns were placed in new 1.5 mL tubes and 30 µL of RNase-free water was added before centrifugation. The 30 µL were then reloaded and centrifuged one last time.

RNA-seq & sequence analysis

Total RNA was sent for sequencing at the Centre d'expertise et de services Génome Québec (Montreal, Canada), where ribosomal RNAs were depleted and messenger RNAs were quantified. RNA-seq raw reads were processed with ShotgunMG⁴⁷. Reads were trimmed and filtered for quality (Trimmomatic v.39) and mapped (BWA v0.7.17) against their respective reference genome. The genome of *B. mycooides*

YL123 originated from our lab, was sequenced, processed and submitted to the NCBI through the Prokaryotic Genome Annotation Pipeline (PGAP) under the assembly accession GCA_024297165.1 on GenBank. The *V. paradoxus* EPS genome was published with accession number NC_014931⁴⁸. BedTools v2.23.0 was used to generate read count files from each .bam file that were then merged to generate a raw count matrix. The latter file was used as entry data for a differential gene expression analysis using DESeq2 (Bioconductor, version 1.36.0). For each strain and each time point, gene expression of samples treated with synthetic miRNAs was compared to those treated with scrambled synthetic miRNAs. The code used for data analysis is on GitHub.

Data availability

Sequence data generated from this study are available on the NCBI portal under accession PRJNA836586.

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 plant species were used in this experiment: the dicotyledon model, *Arabidopsis thaliana* and the monocotyledon model, *Brachypodium distachyon*. An average of 34,119,348 and 33,766,319 raw sequences per sample (**Extended Data Table 1**) 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. The great majority of annotated miRNAs were present in higher proportions in the rhizosphere than in unplanted soils. In many cases, the miRNA sequence was present in the rhizosphere and completely absent in the unplanted soil samples. 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 compared to unplanted soil (**Figure 1 - A**). 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 to unplanted soil (**Figure 1 - B**). Particularly, bdi-miR159b-3p was highly present, along with bdi-miR7757-5p and members of the bdi-miR396 and bdi-miR167 families. Normalised counts of the significantly abundant miRNAs, detected in the rhizosphere of these two plants, were higher in comparison with the counts from unplanted samples (**Extended Data Figure 2 - A;B**), confirming that the majority of plant miRNAs detected were in the rhizosphere samples, with the exception of ath-miR5026. Amongst these significantly abundant rhizospheric miRNAs, nine were common to both *A. thaliana* and *B. distachyon*, and undetected in unplanted soils, suggesting a potential conserved core of miRNAs present in the rhizosphere: miR159b-

3p, miR166a-b-c-d-e, miR167d 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, *e.g.* the same top *A. thaliana* rhizospheric miRNAs (miR158a, miR161.1, miR159a-b-c, miR165b) were identified **Extended Data Figure 2 - D**).

***A. thaliana* 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. Sequences were processed and filtered (**Extended Data Table 1**), then the intra-bacterial putative miRNAs were mapped against *A. thaliana* TAIR10.1 genome, identifying a total of 97 ath-miRNAs. 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 1 - C and Extended Data Figure 2 - C**). To confirm the plant origin of these miRNAs, their sequences were searched for in 3,837 soil bacterial genomes²⁶, of which 1,160 were of bacteria isolated from plants. No matches were found, confirming the detected miRNAs were likely not produced by the bacteria themselves.

Disruption of small RNA biosynthesis in *A. 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 (**Extended Data Figure 3 - C**).

First, 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 (**Extended Data Figure 3 - A**), however, WT plants harboured a lower phyla diversity in their root and rhizospheric communities. The fungal taxonomic profiles varied more across mutants and WT plants (**Extended Data Figure 3 - B**). 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 were common to both bacterial and fungal communities (**Figure 2**) : firstly, PERMANOVA tests indicated that plant type, *i.e.* mutants or WT, explained 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 were 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 and revealed that bacterial diversity was significantly greater in mutant roots and close rhizospheres, compared with WT (**Figure 3 - A**). There was also a significantly higher number of observed fungal ASVs in the distant rhizospheres of mutants compared with WT (**Figure 3 - B**).

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 workflow, which is based on the rules known in plant miRNA-target systems, largely based on the *psRNAtarget*³⁹ online tool. A first global examination of the 428 known ath-miRNAs resulted in 89,229,696 potential target sequences within the 3,837 bacterial genomes described by Levy, *et al.*, in 2018. Amongst these bacteria, those which were isolated from the rhizosphere of *A. thaliana* were selected to create the “ARA” group. Within these genomes, sequences potentially targeted by the 10 ath-miRNAs found in the rhizospheric bacteria (**Figure 1 - C**) were sought. 70,048 sequences were predicted to be targeted by one or more of these intra-bacterial ath-miRNAs (**Extended Data Table 2**). 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. When these far away targets were removed, these 10 ath-miRNAs were still predicted to target 39,389 sequences, in the ARA group. Interestingly, 35,809 potential targets were also identified in the non-plant-associated (NPA) group, composed of 140 bacterial genomes.

These targeted CDSs were categorised within three ontology domains, concerning the function of the gene product (biological process, molecular function) and the cellular component in which the gene product was functional (**Extended Data Figure 4**). The most targeted biological processes, in ARA bacterial genomes, were related to the regulation of metabolic and biosynthetic processes, nucleobase-containing compound metabolism, transmembrane transport, signal transduction and many more. The products of these targeted genes were mostly related to anion, nucleic acid and nucleoside phosphate binding, but also catalytic activities on RNA and DNA, and more. These products are mostly active in the cytoplasm, in membrane protein complexes, in the plasma membrane and in various organelles.

When comparing these functional annotations of targets in ARA and NPA groups, some tendencies were identified (**Extended Data Figure 5**). Certain biological processes are predicted to be more targeted in the ARA group than NPA group, such as cell importation, cellular nitrogen compound and carbohydrate catabolism and monosaccharide metabolism. Molecular functions were also differentially targeted in these two groups. Some functions were further targeted in NPA groups, such as dioxygenase activity, efflux and siderophore transmembrane transportation, whereas hydrolase activity (on glycosyl bonds), organic acid and carbohydrate transmembrane transporter activities were predicted to be more targeted in ARA genomes.

Synthetic plant miRNAs modulate bacterial gene expression

Two bacterial strains were confronted with a mixture of six synthetic miRNAs, the most represented in both *A. thaliana*'s rhizosphere and associated bacteria: miR159a, miR159b, miR159c, miR161.1, miR158a and miR165b. The Gram positive bacterial strain tested was *Bacillus mycoides* YL123, a bacterium isolated from wheat roots⁴⁹. The Gram negative bacterial strain tested was *Variovorax paradoxus* EPS, a root-associated plant growth promoting bacterium⁴⁸, which belongs to the *Variovorax* genus, which is involved in regulating the effect of a whole microbial community on plant growth parameters⁵⁰. *B. mycoides* did not respond to the treatment : no gene was differentially expressed following incubation with the synthetic miRNAs (**Extended Data Figure 6**). In contrast, *V. paradoxus* showed important changes in response to the miRNA confrontation. After 20 min of incubation, the expression of 79 genes was significantly lower in the miRNA-treated cultures and the expression of 44 genes was significantly increased ($P_{\text{adj}} < 0.05$). After 120 min of incubation, the expression of 24 genes was significantly lower in the miRNA-treated cultures and the expression of 104 genes was significantly increased.

The top 25 differentially expressed genes (DEGs), either increased or decreased in response to the miRNA treatment at the two sampling times, are displayed in **Extended Data Tables 3 and 4**. Volcano and MA plots showing DEGs (**Figure 4; Extended Data Figure 7**) reveal that many genes were repressed after 20 min following the addition of the synthetic miRNAs to the bacterial culture, whereas after 120 min, more genes presented an increased expression than decreased. Only one gene was differentially expressed at both time points, a gene coding for a methionine synthase (VARPA_RS01000), which was overexpressed in response to miRNA treatment (**Figure 5**). However, some DEGs belonged to Clusters of Orthologous Genes (COGs) common to both time points, coding for similar gene products within the same functional category. Seven COGs had DEGs after 20min and 120min incubation, mainly related to metabolism, especially lipid metabolism, and transcription functions (**Table 1**). Interestingly, many of these DEGs were first inhibited by the miRNA mix at 20min post-inoculation and then over-expressed after 120min.

Few predicted targets were actually differentially expressed *in vitro*

For further investigation into the mechanisms behind bacterial genes targeted by plant miRNAs, we compared the DEGs from the previous experiment with target genes predicted by our tool, to estimate if similar rules apply to both bacterial and plant systems. The 6 miRNAs used in the previous transcriptomic experiment were predicted to target 237 sequences in the EPS genome. Amongst these, 100 targets were positioned in the "OTHER" category, meaning too far from any CDS, so they were removed from the following analysis, resulting in 137 potential targets. These predicted targets were compared to significantly DEGs in EPS ($P_{\text{adj}} < 0.05$), in response to the 6 synthetic miRNAs. Amongst the 123 DEGs at 20 min, only two were *in silico* predicted as targets : VARPA_RS05960 , coding for a L-iditol 2-dehydrogenase, and VARPA_RS26385, coding for a phosphatidate cytidyltransferase (**Figure 5**). Only two genes were predicted as targets amongst the 128 DEGs at 120min : VARPA_RS00680, coding for a hypothetical protein, and VARPA_RS22555, coding for a non-ribosomal peptide synthetase (**Figure 5**). Interestingly, our bioinformatic tool shows that whilst VARPA_RS05960 and VARPA_RS00680 were targeted by only one miRNA, miR165b, VARPA_RS22555 was targeted by two miRNAs, miR158a-3p and miR159c, and VARPA_RS26385 was targeted by the three miRNAs, miR159a, b and c.

Discussion

Taken together, the results presented here suggest that a hitherto undetected mechanism can contribute to shaping the rhizospheric microbiota. We showed the presence of specific plant miRNAs in the rhizosphere of two model species and their potential uptake by bacteria, as a selection of those miRNAs were found in rhizobacteria. We then demonstrated that a consortium of synthetic rhizospheric miRNAs modulates the gene expression of an essential plant-associated bacterium. Accordingly, plants mutated in small RNA pathways harboured altered microbial communities in their rhizosphere.

We detected plant miRNAs in the rhizospheres of two model 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 processes⁵¹. 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 zones¹¹. By comparing the major miRNAs found in the rhizosphere of *A. thaliana* with those previously found in roots¹¹, all of them were previously 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 zone¹¹. Many root exudates, such as exDNA, soluble compounds and mucilage, are produced and secreted, by border cells, in this region of the root tip⁵². Thus, it is possible that these rhizospheric miRNAs are secreted by roots in the early meristematic zone, at the root tip. Another interesting finding, in both *A. thaliana* and *B. distachyon*, 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 stresses⁵³.

Given our increasing knowledge about small RNAs' function in plant-microbe interactions, it seems relevant that these rhizospheric miRNAs may equally be involved. Interactions *via* small RNAs, including miRNAs, with fungal partners have been covered by previous studies^{13–16,54}, 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. This hypothesis could be further confirmed by using fluorescent synthetic miRNAs combined with confocal microscopy to visualise their entry in bacterial cells. 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 layer⁵⁵. 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 vesicles⁵⁶, it is proposed that bacteria may internalise external DNA *via* vesiduction⁵⁷, *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 *A. thaliana* 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 more 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 methylation⁵⁸. 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 methylation⁵⁹ 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 usually varies in inverse proportion to plant selection, *i.e.* microbial diversity diminishes from bulk soil, to the rhizosphere, and finally, to the endosphere, as the selection strength increases⁶⁰. A high diversity in the roots and rhizosphere of mutants could reflect a weaker selection from these plants, which may be directly or indirectly orchestrated by plant miRNAs and/or siRNAs. As demonstrated in a previous study on the gut microbiota¹⁸, plant miRNAs may have an indirect effect on the microbiota, by impacting the secretion of bacterial metabolites, which, in turn, modifies the growth of surrounding microbes. Anyhow, the mutations addressed in this work are pleiotropic. In order to gain robustness, these results should be supplemented by data which would express a direct link between the miRNAs in the rhizosphere of these mutants and the changes observed in the microbiota. However, we can confirm that disrupting the biosynthesis of small RNAs *in planta* modifies its rhizospheric microbiota.

During our investigation on the impact of plant miRNAs on bacterial transcriptomics, it appeared that *Bacillus mycoides*, a Gram-positive bacterium, did not respond to the treatment like *Variovorax paradoxus*

did, a Gram-negative bacterium. Both Gram-positive and Gram-negative bacteria are able to stabilise sRNA-mRNA interactions *via* chaperone proteins, in the form of RNA-binding Hfq proteins or others such as ProQ or CsrA proteins. Similarly to RISC, these chaperones protect sRNAs and improve the establishment of sRNA-mRNA duplexes. Conversely, there are only a handful of examples of the role of chaperones in sRNA-mediated interactions within Gram-positive bacteria^{61,62}. This ambiguity may partially explain why the two bacteria had very different transcriptomic responses when challenged with synthetic miRNAs. This differential response between *B. mycoides* and *V. paradoxus* could be explained by other factors. On the one hand, this could suggest that the thick peptidoglycan layer of Gram-positive bacteria makes miRNA entry impossible. On the other hand, maybe these bacteria were not competent during our experiment, as it has been shown that some *Bacillus* strains only become competent in response to stress⁶³, which was not the case in these conditions. Another explanation involves plant vesicles, potentially containing RISC proteins, to deliver and enable miRNA entry into bacteria. The lack of these structures in our experiment could explain the absence of DEGs in *B. mycoides* cultures in response to miRNA treatment. However, the *Variovorax* strain did respond to the miRNA treatment, resulting in over a hundred DEGs at both sampling points.

The expression patterns of *V. paradoxus* genes varied between the two time points : after a short incubation period (20 min), most DEGs were repressed by the miRNA treatment, on the contrary, after an extended incubation period (120 min), most DEGs were overexpressed. When analysing DEGs from gene clusters that are both differentially expressed at 20 min and 120 min, it appears that most of them were first repressed and then overexpressed. This could suggest a certain functional compensatory mechanism, in which functions that were repressed by miRNAs would be compensated by other similar genes to restore the lost function. It is also interesting to note that repressed genes after a short incubation period have higher fold changes than those after a longer period, which could indicate a stronger effect of the miRNA treatment, only minutes after the start of incubation. Concerning the function of these DEGs, a majority of them were related to metabolism (carbohydrates, lipids, amino acids...) and transcription. This could mean that in response to surrounding plant miRNAs, bacteria will undergo important shifts in their manner of processing nutrients, by rewiring the transcribed genes, in order to compete or cooperate with the host plant.

In order to understand the mechanisms behind bacterial gene silencing by plant miRNAs, we compared the DEGs from our transcriptomics experiment with our *in silico* predicted target genes. Whilst mechanisms behind miRNA-based interference in plants seem to be consensual, the way eukaryotic miRNAs target and repress bacterial genes has to be determined. Plant miRNAs inhibit target gene expression *via* mRNA cleavage or translation repression, through near perfect sequence complementarity⁶⁴. Hypotheses from previous research were based on the assumption that eukaryotic miRNAs target bacterial genes in the same manner, through sequence complementarity, and were somewhat confirmed through *in vitro* and *in vivo* experiments^{17,18}. 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*³⁹. Our predictions suggest that the 10 ath-miRNAs internalised in

rhizobacteria target many genes, in both *A. thaliana* rhizospheric bacteria and non-plant-associated bacteria. However, very few predicted targets were actually differentially expressed in our *in vitro* conditions. In our case, only 4 out of 251 actually differentially expressed genes were predicted to be targeted by at least one of the six synthetic miRNAs used in the treatment. This low predictability rate could be explained by three possibilities: (i) plant miRNAs do not target bacterial genes in the same way they target plant genes; most if not all scoring schemes between miRNA and their targets have been developed in the context of miRNA *versus* plant genome interactions. It is possible that the interaction between miRNA and bacterial genomes follow different rules. (ii) the observed DEGs result from a cascade of translational shifts originating from a select few targeted genes and/or (iii) target genes are translationally repressed, which would be undetectable with transcriptomics. This translational repression is, at least in plants, less common than mRNA cleavage⁶⁴. It should also be noted that some miRNAs protect targets from repression, notably in the case of arbuscular mycorrhizal fungi symbiosis⁵⁵, in which case the target gene would not be differentially expressed. It is also possible that some of the DEGs observed in *V. paradoxus* result from miRNA side effects such as the production of secondary siRNAs, resulting from the slicing of target genes, which in turn can silence other target genes⁶⁶. Notably, miR165b and miR161.1 from our experimental miRNA mix are considered siRNA-triggering miRNAs⁶⁶, meaning their effect can be much broader than their target gene. This would explain a higher number of DEGs compared with the number of predicted miRNA targets, especially seeing that two out of the four DEGs were predicted to be targeted by miR165b. Another “side effect” of miRNAs could exist when transcriptional factors are targeted, such as COG0583, corresponding to the LysR family, which was strongly repressed after 20 min incubation with miRNAs, then overexpressed after 120 min. Even though very few targets were actually differentially expressed in response to the addition of miRNAs to the culture, our study showed that plant miRNAs strongly affect transcriptomics of an important plant-associated bacterium, *V. paradoxus*, but not *B. mycoides*, indicating a selective gene-silencing mechanism in the rhizosphere.

There are still many unanswered questions concerning the function of rhizospheric plant miRNAs and their impact on the rhizomicrobiome. Future work is required to understand the active or passive processes explaining the presence of plant miRNAs in the rhizosphere. Further investigation is necessary to comprehend more deeply the effect of rhizospheric miRNAs on the composition and especially the activity of the rhizospheric microbiota. This work focused solely on potential plant-microbe interactions in the rhizosphere, due to the novel nature of this microbial regulation mechanism, however, it is also possible that these rhizospheric miRNAs target surrounding plants, as described by Betti, *et al*⁶⁷. Nevertheless, for the first time, we detected plant miRNAs in the rhizosphere and showed that they were also found inside rhizospheric bacterial cells, affecting gene expression in some bacteria, thereby providing a mechanism to shape the rhizosphere microbiota. Our study suggests a completely novel way that plants shape their rhizospheric microbiota, challenging the current paradigm of rhizosphere microbial assembly. This knowledge will be essential for future efforts to engineer the plant microbiota.

Declarations

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Authors' contributions

AE, EY, and CM conceived and designed research. VD, HM and JD extracted microRNA from the rhizosphere, VD and HM carried out *Arabidopsis* mutants analysis and rhizomicrobiome isolation. HM performed and interpreted microbiome diversity. EC, JT and HM carried out and interpreted bioinformatic analysis. HM, EY, AE and CM wrote the manuscript. AE, EY and CM acquired funding.

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare that they have no competing interest

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Table

Table 1: Common Clusters of Orthologous Genes (COGs) with differentially expressed genes in both time points, showing which functional categories of *V. paradoxus* EPS are affected by the presence of rhizospheric miRNAs. Log₂FC is the log₂-transformed fold change, representing change in gene expression in miRNA-treated cultures in comparison with the control cultures treated with scrambled miRNAs. The values in bold are related to an identical gene. Orange and green arrows represent the decrease and increase induced by the miRNA treatment, respectively.

COG accession number	log2FC at 20min	log2FC at 120min	Gene product	Functional category
COG0183	 -4,69 (VARPA_RS25660) -1,67 (VARPA_RS04385)	 0,52 (VARPA_RS10780)	Acetyl-CoA acetyltransferase	Lipid metabolism
COG0318	 -1,35 (VARPA_RS04355)	 -0,52 (VARPA_RS22025)	Acyl-CoA synthetases (AMP-forming)/AMP-acid ligases II	Lipid metabolism / Secondary metabolites biosynthesis, transport, and catabolism
COG0583	 -3,4 (VARPA_RS22520)	 0,35 (VARPA_RS11995)	LysR transcription factor	Transcription
COG1028	 -2,7 (VARPA_RS05960)	 0,97 (VARPA_RS03770)	Dehydrogenases	Secondary metabolites biosynthesis, transport, and catabolism / General function prediction only
COG1410	 0,38 (VARPA_RS01000)	 0,43 (VARPA_RS01000)	Methionine synthase I, cobalamin-binding domain	Amino acid transport and metabolism
COG1960	 -1,93 (VARPA_RS04375) -1,82 (VARPA_RS04380) -0,82 (VARPA_RS17425)	 0,93 (VARPA_RS11970)	Acyl-CoA dehydrogenases	Lipid metabolism
COG2814	 0,38 (VARPA_RS15715)	 -0,42 (VARPA_RS29615)	Arabinose efflux permease	Carbohydrate transport and metabolism

Figures

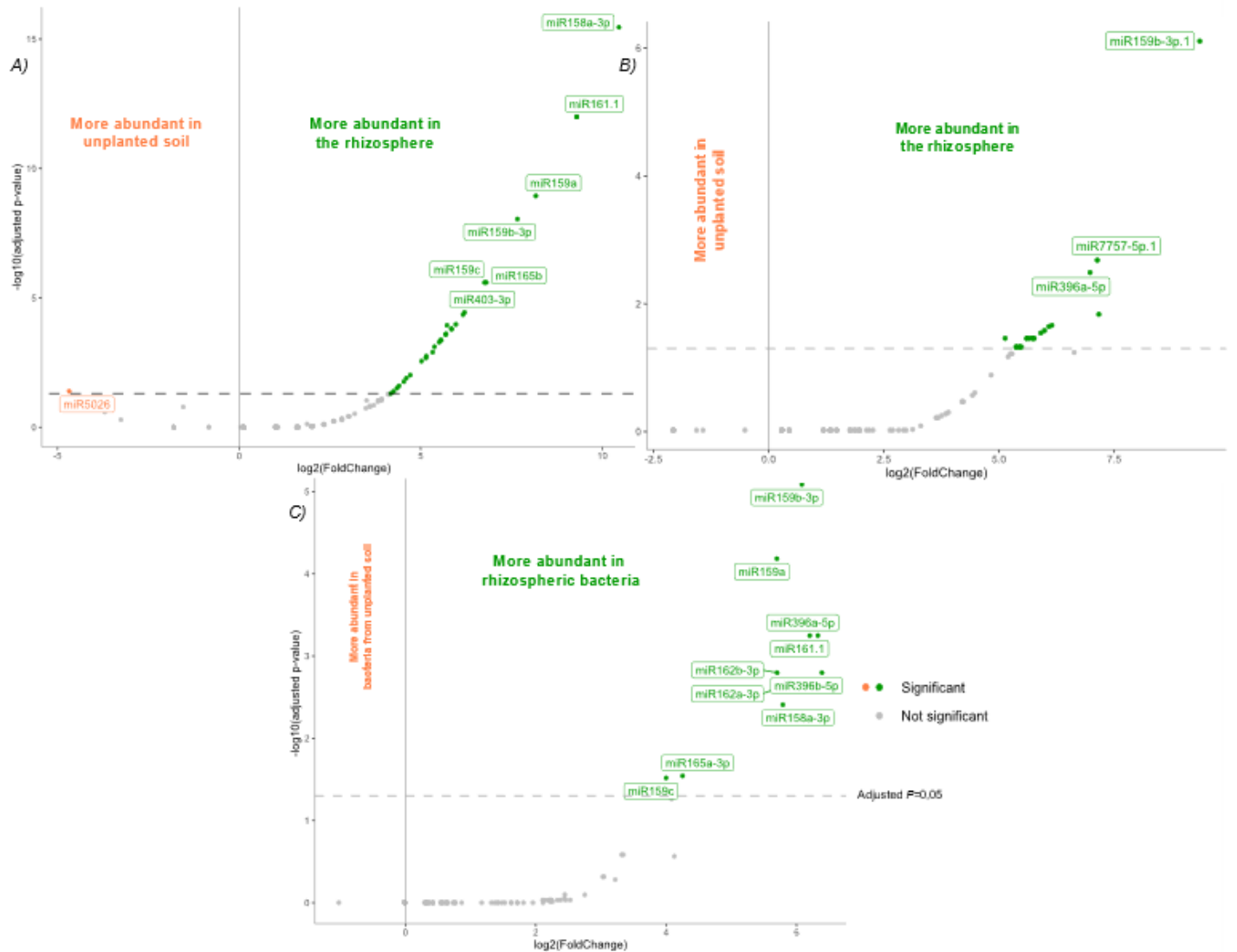


Figure 1

Arabidopsis thaliana miRNAs (A) and *Brachypodium distachyon* miRNAs (B) identified in their respective rhizospheres. Differentially abundant miRNAs found in the rhizosphere of each plant and unplanted soil, resulting from DESeq2 analysis. In *A. thaliana*'s rhizosphere, twenty-nine miRNAs were significantly more abundant than in unplanted soil (points on the right). Whereas twenty miRNAs were significantly more abundant in the rhizosphere of *B. distachyon* than in unplanted soil. *A. thaliana* miRNAs were also identified in bacteria isolated from rhizospheric and unplanted soil samples (C). All detected miRNAs are represented; however, many had the same Fold Change and P -value, meaning their dots are stacked. Statistical analysis was performed using a Wald test, with a parametric fit. P -values were adjusted using the Benjamini & Hochberg method (*A. thaliana* : $n = 3$; *B. distachyon* : $n = 2$ rhizosphere samples, $n = 3$ unplanted soil samples; Bacteria : from the rhizosphere, $n = 8$, and unplanted soil, $n = 6$).

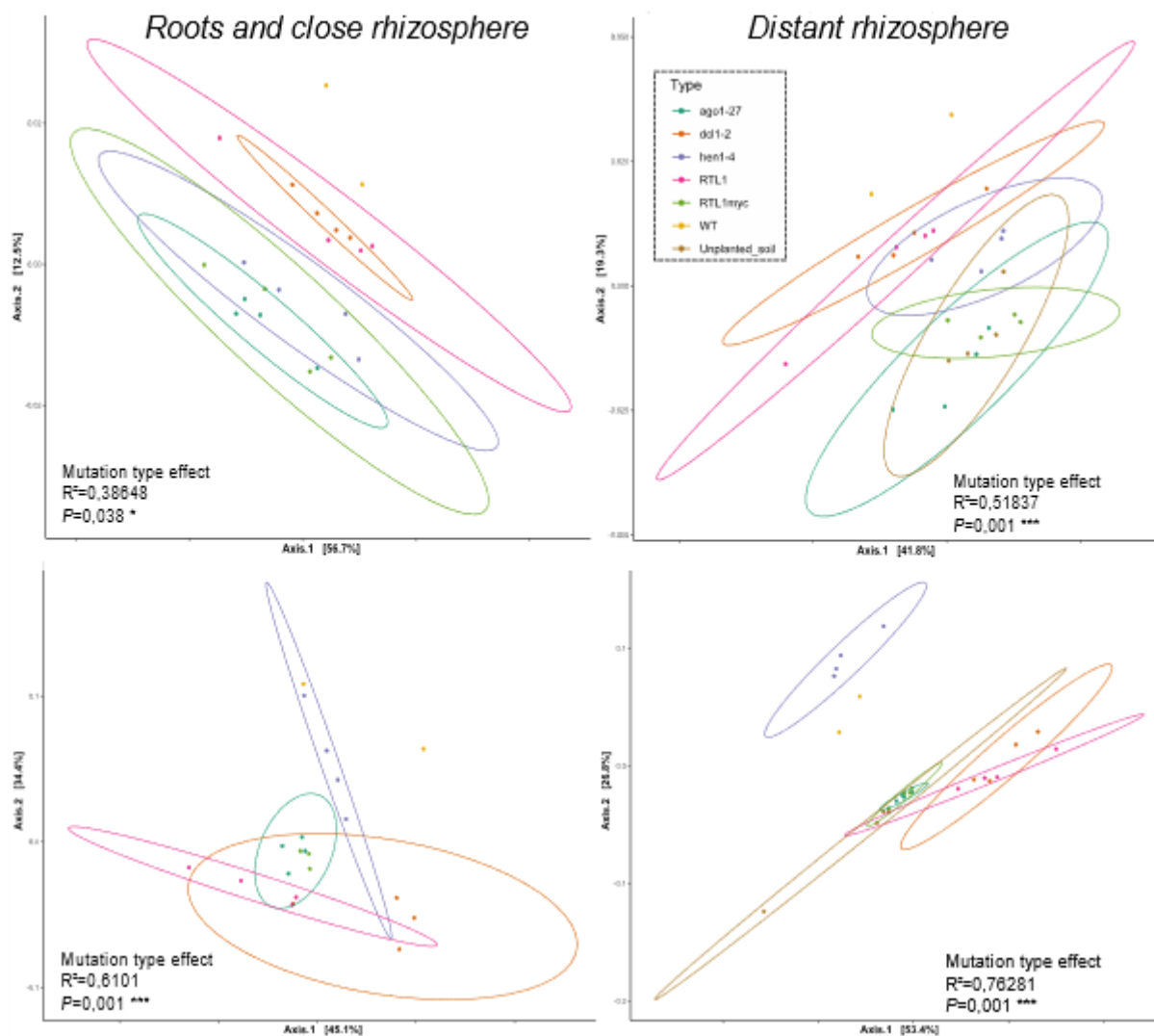


Figure 2

Principal Coordinate Analysis (PCoA) ordination, using a Weighted UniFrac distance matrix, of root and rhizospheric bacterial (A) and fungal (B) communities. Statistical analysis was performed using a PERMANOVA test on the distance matrices (adonis function) : $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***.

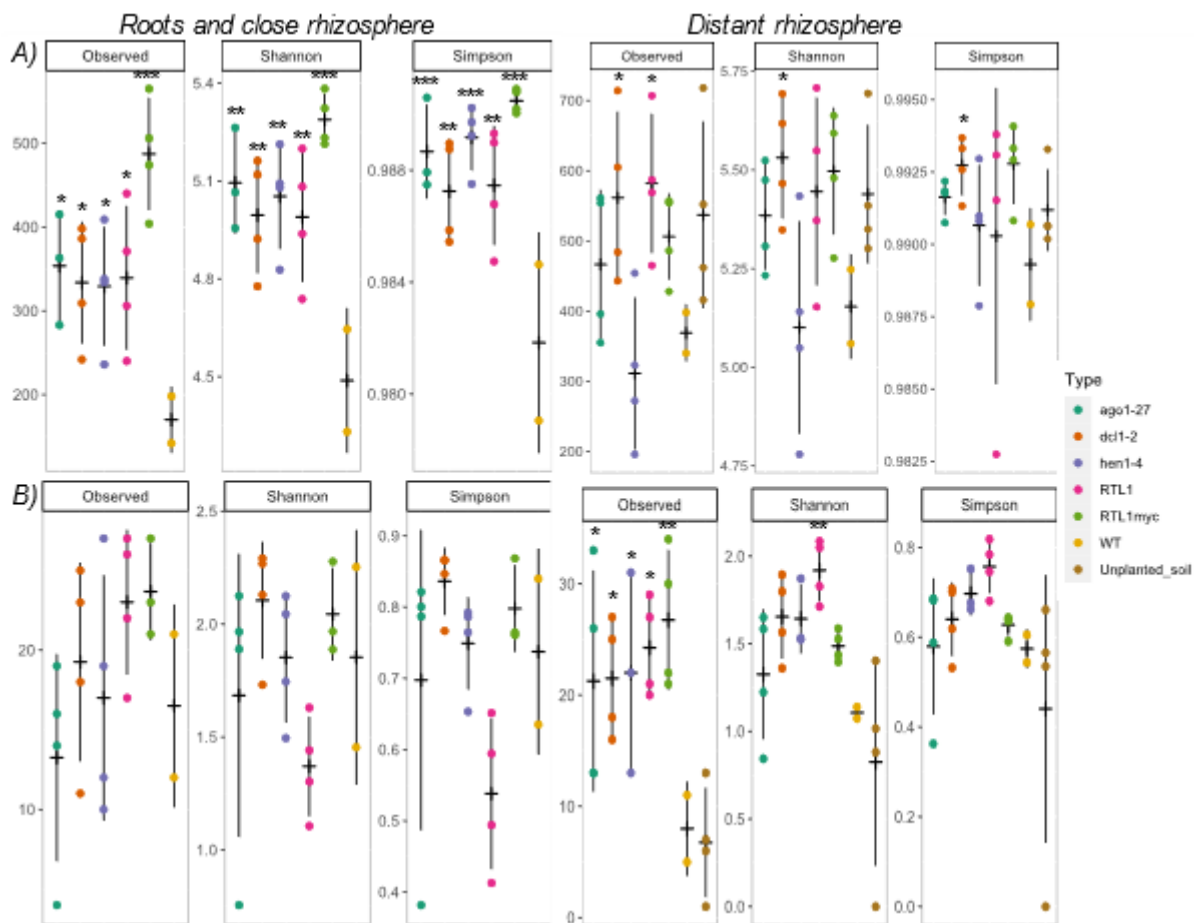


Figure 3

Alpha diversity of root and rhizospheric bacterial (A) and fungal (B) communities: observed number of ASVs, Shannon's index and Simpson's index. The centre line represents the mean. Statistical analysis was performed using a linear model or a general linear model, if residuals were not normal ($P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***).

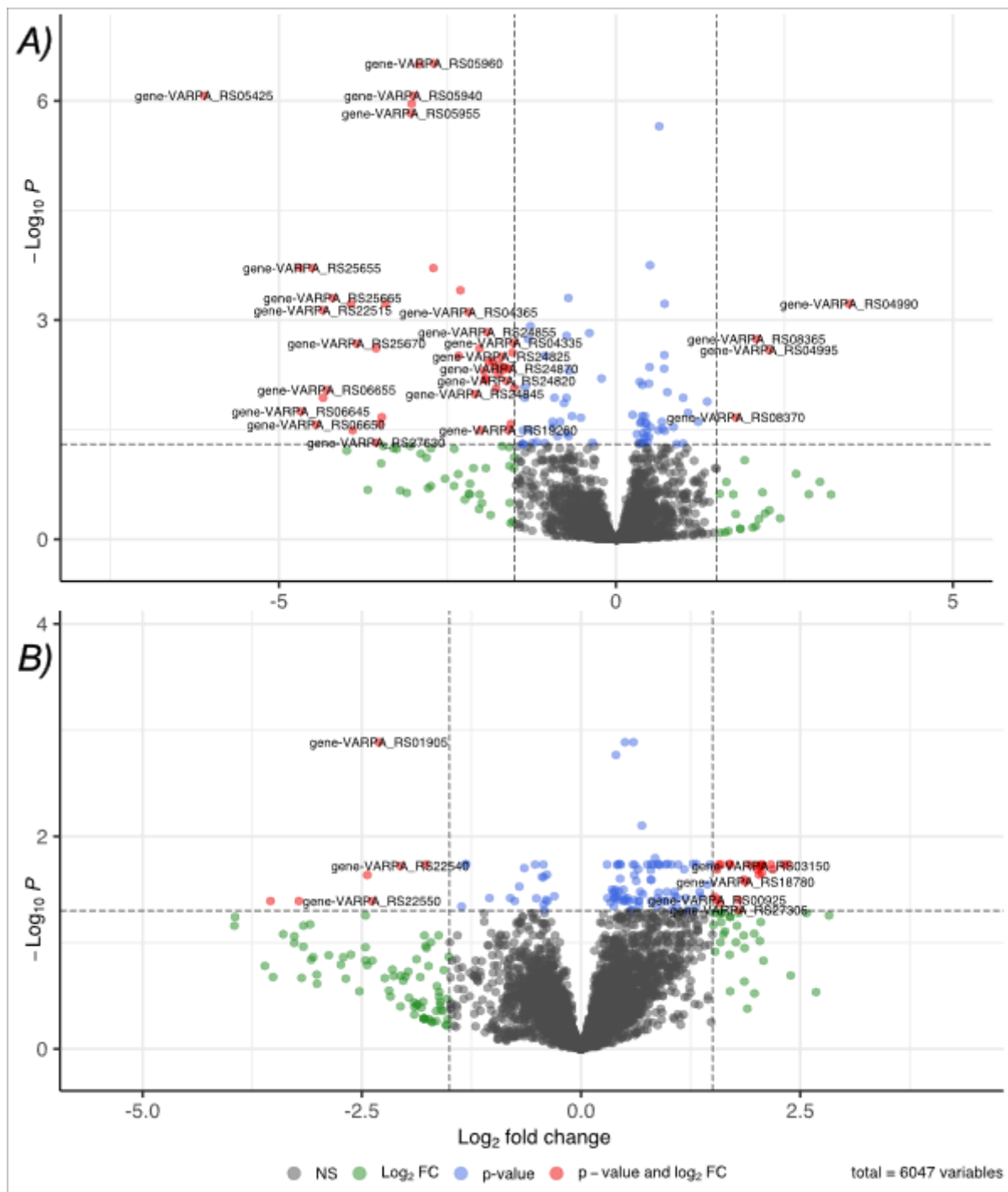


Figure 4

Differentially expressed genes in *Variovorax paradoxus* EPS after 20 min (A) and 120 min (B) of incubation with six synthetic rhizospheric miRNAs (miR158a, miR159a, miR159b, miR159c, miR161.1, miR165b). Fold change threshold is |1.5| and *P*-value was adjusted with Benjamini-Hochberg and the threshold is 0.05.

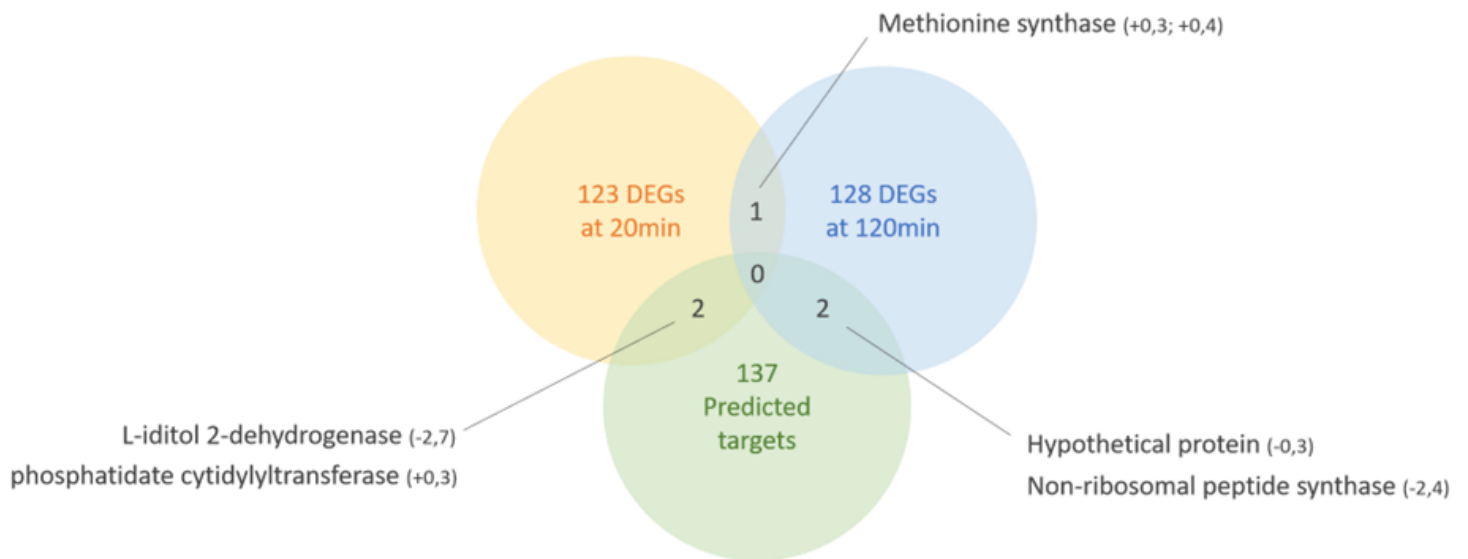


Figure 5

Common genes (based on VARPA locus) both predicted as potential targets, in *Variovorax paradoxus* EPS genome, and differentially expressed after 20 min or 120 min of incubation with six rhizospheric miRNAs. Numbers in parentheses are the log2FoldChange values corresponding to differential expression at 20 min or 120 min. DEGs = Differentially Expressed Genes ($P_{adj} < 0.05$).

Supplementary Files

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