



Supplementary Information for:

The ectomycorrhizal fungus *Pisolithus microcarpus* encodes a microRNA involved in cross-kingdom gene silencing during symbiosis

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This PDF file includes:

Supplementary text
Figures S1 to S4
Table S1
Legends for Dataset S1 to S7
SI References

Other supplementary materials for this manuscript include the following:

Datasets S1 to S7

Supplementary Text

Detailed Methods

Plant and fungal materials

E. grandis seeds were obtained from the Commonwealth Scientific and Industrial Research Organization (CSIRO, Clayton, VIC Australia) tree seed center (Seedlots: 20974 & 21068). Following the method previously described in Wong et al. (1), *E. grandis* seeds were sterilized, germinated on 1% water agar for a month, and then transferred to ½x modified Melin-Norkrans (½MMN) media with the root system covered by a piece of porous cellophane membrane for another month of growth. Four isolates of the model ECM fungal species *P. microcarpus* (isolates SI9, SI14, R4, R10; 2) were used to identify novel miRNAs, while only two of these were used to profile their expression patterns across colonization (isolates SI14 and SI9; Figure 1A,B) and only one isolate was used to characterize *Pmic_miR-8* (isolate SI14). Prior to sub-culturing for the experiments described below, cultures were maintained on 1x MMN media in a dark cabinet with temperature kept at a constant 25°C.

ECM fungi-eucalypt interaction time-course

A time-course of colonization between *P. microcarpus* isolate SI14 or SI9 and *E. grandis* was used to profile the expression of identified miRNAs over the course of colonization. Briefly, two-month-old *E. grandis* seedlings were directly inoculated with *P. microcarpus* by placing roots of the seedlings in direct contact with the growing front of two-week-old fungal cultures growing on ½ MMN media, and then kept in a controlled growth chamber (22–30°C night/day temperature; 16 h light cycle). After a fixed time interval of inoculation (24h, 48h and 2 weeks (2w)), roots and attached fungal mycelia were sampled as were sterile roots of uninoculated *E. grandis* seedlings (control) and axenically grown free-living mycelia (FLM) grown in monoculture harvested as a control. In addition, a 24hr pre-symbiotic interaction (pre-symbiosis) setup was also prepared according to Wong et al., (1), where the host root and fungal mycelia were physically separated by a solute-permeable membrane (Kleerview Covers by Fowlers Vacola Manufacturing Co Ltd.; Figure S1). We verified the inability of *P. microcarpus* hyphae to penetrate these membranes using scanning electron microscopy. Samples of cellophane

membranes upon which fungi had been growing were carefully removed from the growth medium and cut into sections ($\sim 10 \times 10$ mm) and mounted on conductive carbon tape. The samples for Figure S1C were mounted in a curved manner to enable simultaneous visualization of the bottom of the membrane as well as a cross-section to observe the fungal growth on the membrane. The samples were imaged with a scanning electron microscope (model JEOL JSM 6510LV, JEOL, Japan) in low vacuum mode at 30 Pa and 10 kV accelerating voltage with a working distance of 19 mm for imaging. To further verify no fungal disruption of the membrane, we used a scalpel blade to carefully remove the fungi from the surface of the membranes and repeated the imaging of the membranes (Figure S1E) and compared this to membranes with no fungal growth (Figure S1D). In both cases, no holes or disruptions to the membrane were observed. Therefore we are confident that *P. microcarpus* in this pre-symbiosis experimental setup did not physically interact with the plant root.

Small RNA library preparation and sequencing procedures

All root and fungal samples were snap-frozen in liquid nitrogen and RNA extraction was performed using the ISOLATE II miRNA kit (Bioline) as per manufacturer's instruction. RNA samples of four biological replicates each for six different conditions (*E. grandis* axenic control, FLM, pre-symbiosis, 24h, 48h, and 2 weeks) were extracted for isolates SI14 and SI9. The small RNA-sequencing (sRNA-seq) was performed at the Joint Genome Institute (JGI, Berkeley, California). Plate-based small RNA sample prep was performed on the PerkinElmer Sciclone NGS robotic liquid handling system using Illumina's TruSeq Small RNA Library Prep Kit and following the protocol outlined by Illumina in their user guide: http://support.illumina.com/sequencing/sequencing_kits/truseq-small-rna-kit.html, and with the following conditions: total RNA starting material of 1 ug per sample, 11 cycles of PCR for library amplification, and size-selection of miRNA and other small RNA library templates on the Coastal Genomics Ranger instrument. The prepared libraries were quantified using KAPA Biosystem's next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. Sequencing of the Illumina flow cell was performed on the Illumina NextSeq500 sequencer using NextSeq500 High-Output 75 cycle kits, v2, following a 1x75 indexed run recipe.

Small RNA-seq data preprocessing and in silico miRNA identification

From the generated raw reads, BBDuk (<https://sourceforge.net/projects/bbmap/>) was used to evaluate artifact/adaptor sequences by kmer matching, allowing 1 mismatch and detected artifact/adaptors were trimmed from the 3' end of the reads. RNA spike-in reads, PhiX reads and reads containing any Ns or low-quality reads were removed. Finally, following trimming, reads under the length threshold were removed (minimum length 17 bases). Filter parameters for smRNA are: rna=t trimfragadaptor=t qtrim=r trimq=6 maxns=1 maq=10 minlen=17 mlf=0 khist=t trimk=23 mink=3 hdist=1 hdist2=1 ktrim=r sketch mito chloro ribomap taxlevel=species. Reads were filtered to exclude reads containing exact matches to either Ribosomal (*E. grandis*, *P. microcarpus*, *P. albus* from the SILVA database) or Chloroplast (*E. grandis*: RefSeq NC_014570.1) 17-mers using bbdduk. Finally, reads longer than 40bp were discarded.

Using the reference genome of *E. grandis*

(https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Egrandis) and *P. microcarpus* 441 (<https://mycocosm.jgi.doe.gov/Pismi1/Pismi1.home.html>), ShortStack was run for each library in order to identify fungal-specific sRNA producing loci (or "Clusters"), and to identify putative miRNA genes *de-novo* (3,4). ShortStack will *de-novo* identify clusters of small RNA accumulation genome-wide. The small RNA-forming loci (or clusters) are identified based on the coverage pattern of reads and read length. Additionally, Shortstack uses a set of 15 criteria based on RNA-folding to classify miRNA loci. The loci would not be annotated as miRNA if:

1. no reads at all aligned in locus
2. < 80% of reads in the Dicer size range between 20 and 24nt. (DicerCall is a number that indicates the predominant size of the RNA population from the locus.)
3. Major RNA abundance was less than 2 reads.
4. Major RNA length is not in the Dicer size range between 20 and 24nt.
5. Locus size is > than maximum allowed for RNA folding, which is 300 nt
6. Locus is not stranded (>20% and <80% of reads aligned to top strand)
7. RNA folding attempt failed at locus
8. Strand of possible mature miRNA is opposite to that of the locus
9. Retrieval of possible mature miRNA position failed

10. General failure to compute miRNA-star position
11. Possible mature miRNA had > 5 unpaired bases in predicted precursor secondary structure.
12. Possible mature miRNA was not contained in a single predicted hairpin
13. Possible miRNA/miRNA* duplex had >2 bulges and/or >3 bulged nts
14. Imprecise processing: Reads for possible miRNA, miRNA-star, and their 3p variants added up to less than 50% of the total reads at the locus.
15. Maybe: Passed all tests EXCEPT that the miRNA-star was not sequenced. INSUFFICIENT evidence to support a de novo annotation of a new miRNA family.

Only miRNA that passed all tests including sequencing of the exact miRNA-star is considered a de novo annotation of a new miRNA family (3). Loci covered by read-clusters from at least 10% of all libraries were considered for quantification across libraries. Clusters for which no DicerCall was reported in any sample were removed. These regions were expanded to a minimum length of 75bp around their center and quantified in a second ShortStack run (in "--nohp" mode), using the alignments generated for the single libraries. Finally, loci classified as miRNA in the single libraries were merged across libraries (again specifying the "--nohp" option in ShortStack).

Differential miRNA expression profiling during ECM colonization

Sequences of the novel miRNA loci identified above were used as references for mapping and quantification across all sRNA libraries representing different time-points of ECM colonization using bowtie with the following parameters: -v 1 -m 50 -a --best --strata (version 1.2; 5). Within these parameters:

- -v 1 : allows only 1 mismatch
- -a --best --strata: report only those alignments in the best alignment "stratum". The alignments in the best stratum are those having the least number of mismatches
- -m 50: refrain the tool from reporting any alignments of reads that have more than 50 alignments on genome.

The count table of fungal sRNA libraries were then used as input for normalization and differential expression analysis using the R-based “DESeq2” package (version 1.24.0; 6). Heatmaps were generated with the fold change values of differential expressed miRNAs ($|\log_2(\text{fold change})| > 1$; adjusted p-value < 0.05) using Heatmapper (7).

Pre-symbiotic methodology to identify cross-kingdom sRNA transfer between P. microcarpus and E. grandis

Two-month-old *E. grandis* seedlings were placed into pre-symbiotic interaction with two-week-old fungal mycelia of the four isolates of *P. microcarpus* mentioned above. After one week of pre-symbiotic interaction, three to four *E. grandis* root systems per fungal isolate were sampled and frozen in liquid nitrogen. Following the manufacturer’s instructions, the small RNA fractions were extracted from the pre-symbiotic root tissues using ISOLATE II miRNA kit (Bioline), pooled, and then used for small RNA sequencing at the JGI. The accession numbers associated with the generated small RNA libraries can be found in Dataset S6. Preprocessing of raw reads were done as described above. The filtered reads were then mapped against the *P. microcarpus* and *E. grandis* genome using bowtie with conditions as described above. The read count of each unique sequences mapped to *P. microcarpus* was recorded (Dataset S1). These *P. microcarpus*-aligned reads were subsequently mapped against the aforementioned novel *P. microcarpus* small RNA clusters that were identified with ShortStack. The genome locations and read counts of these small RNA clusters can be found in Dataset S2. From these analyses, four fungal miRNAs were found to have normalized counts >1 in RNA extracted from pre-symbiotic root tissue of *E. grandis*. To further ensure that these four miRNAs were fungal in origin, and not encoded by the plant, we searched for similar sequences using the NCBI BLAST webserver (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to search against the nucleotide collection of *E. grandis* with Megablast algorithm with default setting. No significant hits were identified. Further, to verify the reproducibility of the results presented in Figure 1C, we extracted RNA from three biological replicates of *E. grandis* axenic roots (i.e. negative control) or three biological replicates of *E. grandis* pre-symbiotic roots (with isolate SI14) and ascertained whether we could identify the presence of *Pmic_miR-2, 3, 8, 9* using the stem-loop cDNA generation and QPCR methods described below. We also included two other miRNAs, *Pmic_miR-6, 10* which had not been found in the pre-symbiotic roots based on Illumina sequencing, but were induced by the

colonization process in the fungus, to act as a negative control for this experiment. To quantify the copy number of each miRNA, we PCR amplified each of the miRNAs, purified them using the Wizard SV Gel and PCR Clean-Up system (Promega) according to manufacturer's instructions and generated a standard curve with known numbers of copies to relate transcript copy number to Ct in our QPCR cycling.

Pmic_miRs target prediction

The putative *E. grandis* and *P. microcarpus* target mRNA sequences of *Pmic_miRs* were identified using a small RNA target prediction algorithm—psRNATarget (version 2.0; 8). The sequence of *Pmic_miRs* were searched against the *E. grandis* and *P. microcarpus* 441 transcript libraries using the default V2 scoring schema: (i) no more than 2 mismatch at the seed region between 2nd and 13th nucleotides other than U:G pairs, (ii) penalty for mismatches other than U:G pair = 1, (iii) penalty for opening gap = 2, and (iv) penalty for opening gap = 2. Upon identifying putative targets, the analysis pipeline also included an evaluation of the expression pattern of the putative targets whereby the expression patterns of *Pmic_miRs* must co-vary with their putative target. Additionally, a cutoff of expectation value <3 has been applied to select the most plausible target mRNA sequences. Using these criteria, only putative targets in *E. grandis* were identified as all putative targets in *P. microcarpus* had expectation values >3.

Whole-mount in situ hybridization of Pmic_miR-8 and confocal imaging

Two-month-old *E. grandis* seedlings were either set up into a pre-symbiotic interaction or left to grow axenically without fungus for one week. After this time, root tips were excised and vacuum infiltrated with 4% paraformaldehyde for 15 minutes and then left overnight at 4°C. A subset of samples were treated for 15 minutes in RNase at room temperature, followed by three washes in 1x PBS (pH 7.4) prior to fixation to remove/reduce the possibility of external miRNA, or RNase treated for 15 minutes followed by fixation and then a secondary treatment with RNase post-fixation and permeabilization to remove external and internal bound RNA, while another subset of roots were plasmolysed in 1.5M sucrose prior to, and during, fixation. Following fixation, all samples were rinsed in 1x PBS (pH 7.4) three times for 5 min intervals. Samples were then treated using 20 µg/mL Proteinase K and 0.5% Triton-X in TE buffer for 60 min at 37°C to digest cellular RNases and protein excess and to permeabilize the cells for probe entry.

Samples were again rinsed in 1x PBS (pH 7.4) three times for five-minute intervals followed by incubation in 0.2% glycine at room temperature for 5 minutes to stop protease activity. Following glycine treatment, samples were post-fixed with 4% paraformaldehyde for 5 min and again rinsed in 1x PBS buffer. During the rinsing steps, the anti-*Pmic_miR-8* or scrambled LNA probes with a FAM fluorophore modification (35 μ M, sequence detailed in Table S1) was denatured in 50% formamide at 85°C for three minutes followed immediately by ice for 5 min. Either 20 μ L of these solutions, or 20 μ L of 50% formamide as a probe-free control, were added to the tubes containing the root tips along with 80 μ L of hybridization buffer (50% formamide, 25% dextran sulfate, 0.5% SDS, 1.25x Denhardt's solution, 0.5M NaCl, 25 mM Tris-HCl (pH 7.5), 12.5 mM sodium phosphate (pH 6.8), 12.5 mM EDTA, 1.25 mg/mL tRNA) and incubated at 50°C overnight with shaking at 300 rpm. The following day, the samples were placed through a series of high stringency washes to remove unbound probes. Initially the samples were rinsed twice for 30 min each at 50°C with 0.2x SSC buffer (30 μ M NaCl, 3.5 μ M Na₃C₆H₅O₇, pH 7.0) followed by an additional five-minute wash in 0.2x SSC for 5 minutes at 37°C and a final wash with 0.2x SSC for five minutes at room temperature. The final two wash solutions contained 0.1% propidium iodide to stain nuclei. The propidium iodide was de-stained using a final buffer rinse and the roots were mounted on glass slides and observed using an inverted Leica SP6 confocal microscope. The LNA probe fluorophore was excited at 488 nm. All samples were imaged using the same laser intensity/gain and emission spectrum to ensure comparability between the samples.

Synthetic RNA treatments and ECM colonization assays

Two-month-old *E. grandis* seedlings were directly inoculated with SI14 using the setup mentioned previously and left to begin the colonization process for one week. After seven days, a minimum of 6 plants per treatment were sprayed with nebulized synthetic sRNA of different sequences daily for a further seven days (100 μ l of a 20 nM sRNA solution per plant using MAD Nasal Intranasal Mucosal Atomization Device; Teleflex). The delay in spray treatment was based on the profiled expression of *Pmic_miR-8* which identified that it was only significantly induced in the late-stages of colonization between *P. microcarpus* SI14 and *E. grandis*. These synthetic sRNAs included single and double-stranded *Pmic_miR-8* (customized sequences to be identical to the mature form of *Pmic_miR-8*), *Pmic_miR-8** (the complementary strand to

Pmic_miR-8), an antisense *Pmic_miR-8* inhibitor synthesized with a 2'OMe RNA backbone and with a ZEN modification to inhibit denaturation from the mature *Pmic_miR-8*, an antisense *Pmic_miR-8* with a two base mis-match in positions 10 and 11 to stop the cleavage by ARGONAUTE, as well as two scrambled sequences to act as a negative controls, one scrambled sense (Integrated DNA Technologies) and one antisense scrambled sequence. Sequences of the customized sRNAs are detailed in Table S1. On the 14th day of colonization (seventh day of sRNA treatment), four hours after the final spray treatment, the ECM colonization rate was scored (number of root tips showing mantle formation vs. total roots in direct contact with the fungal mycelium) as were the number of senesced mycorrhizal root tips (i.e. roots showing initial mantle formation followed by root outgrowth). For microscopic analysis, roots were preserved in 4% paraformaldehyde and observed with fluorescence microscopy as previously described to assess the Hartig net formations (9). Briefly, preserved mycorrhizal root tips were washed three times in 1X PBS buffer and then embedded in 6% agarose (w/v ddH₂O). 30µm-thick transverse cross-sections were sliced from the embedded roots using a vibrating microtome (model 7000smz-2; Campden Instruments Ltd.). The sections were then stained in 0.1% w/v Wheat Germ Agglutinin (WGA; Lectin from *Triticum vulgare* FITC conjugate, Sigma-Aldrich) in 1X PBS buffer for 10 min, and then with 0.1% (w/v in 1X PBS buffer) propidium iodide for another 10 min before washing in 1X PBS buffer for three times. Sections were then observed and imaged using an inverted Leica TCS SP5 laser scanning confocal microscope with the following setting: excitation at 488nm, 496nm and 561nm (20% power), emission collected at 515-530nm for WGA and 600-650nm for propidium iodide. Each fluorescent image was examined with ImageJ where the Hartig net depth was measured for each root section.

Quantitative PCR expression validation of Pmic_miRs

Mycorrhizal root tip samples from either the timecourse of colonization or following 1 week of spray treatment with synthetic miRNAs were snap frozen in liquid nitrogen and their RNA extracted using the Spectrum Plant Total RNA kit (Sigma-Aldrich) following the procedure for recovery of sRNAs. The difference in RNA extraction kits was due to the previous kit being discontinued. RNA transcript quantitation by QPCR was used to verify that each miRNA was differentially regulated as expected using a stem-loop reverse transcription QPCR approach as described previously (10). Briefly, total RNA (1 µg) including sRNA was used for generation of

target-specific cDNA using the stem loop primers as shown in Dataset S7. The reaction mix containing DNase I-treated RNA, RNase-free H₂O, and 1 μ L stem-loop RT primer (1 μ M) was heated to 65 °C for 5 minutes, incubated immediately on ice for 2 min before the addition of 4 μ L 5X first-strand buffer, 2 μ L DTT (0.1 μ M), 0.25 μ L Superscript III reverse transcriptase (ThermoFisher Scientific, Cat# 18080-044), and 0.1 μ L RNaseOUT 458 (ThermoFisher Scientific, Cat# 10777-019). A pulsed RT was performed (30 min at 16 °C, followed by 60 cycles at 30 °C for 30s, 42 °C for 30 s and 50 °C for 1 s) and Q-CR performed using 5x diluted cDNA and Bioline SensiFast no ROX QPCR mix using the universal primer and gene specific primers as shown in Dataset S7. At the end of each QPCR run, a dissociation curve assay (from 95 °C to 65 °C) was performed to ensure the specificity of each reaction. A total of three biological replicates per treatment were used to determine expression levels of each *Pmic_miR*. Relative expression was determined using the $2^{-\Delta\Delta CT}$ method whereby axenically grown *P. microcarpus* SI14 was used as a control for the timecourse, or mycorrhizal tissues treated with the scrambled sRNA (either sense or antisense scrambled inhibitor) were used as the control tissues for tissues treated with synthetic miRNAs to supplement or inhibit *Pmic_miR-8*, respectively.

Pmic_miR-8 putative target expression verification

To determine which putative host gene may be influenced by *Pmic_miR-8* as predicted by psRNATarget as described above, we took the total RNA extracted from *ss* and *dsPmic_miR-8* or *asmiR-8* with a *ZEN* modification (i.e. *asZEN* in figures) or *asmiR-8-Bulge* (i.e. *asBulge* in figures) inhibitor treated tissues as described above, as well as the scrambled controls, and generated cDNA using the Tetro cDNA synthesis kit (Bioline) as per manufacturer's instructions and using only the oligo-dT primer. Using the SensiFAST SYBR no-ROX Q-PCR kit (Bioline) following manufacturers' instructions, we then analyzed the expression patterns of the top three putative target genes of *Pmic_miR-8*: *Eucgr.K00246*, *Eucgr.L01882*, and *Eucgr.E03170*. Two internal control genes (*Eucgr.C00350* and *Eucgr.K02046*) were used to normalize the results. Given the large gene family to which *Eucgr.E03170* belongs, we also did a homology search for homologues that had a similar binding site for *Pmic_miR-8*. Based on homology to *Eucgr.E03170*, we identified three hits with high homology: *Eucgr.E03194*, *Eucgr.E03196*, *Eucgr.E03203*. We also tested the expression for these genes in the same tissues (Fig 3K; Figure

S3). All primers sequences can be found in Dataset S7. A total of three biological replicates per treatment was used to determine expression levels of each gene. Relative expression was determined using the $2^{-\Delta\Delta CT}$ method whereby tissues treated with the scrambled sequences (either ssScrambled or asScrambled) were used as the control tissues for tissues treated with *Pmic_miR-8* supplementation or *Pmic_miR-8* inhibitors, respectively.

SI Figures:

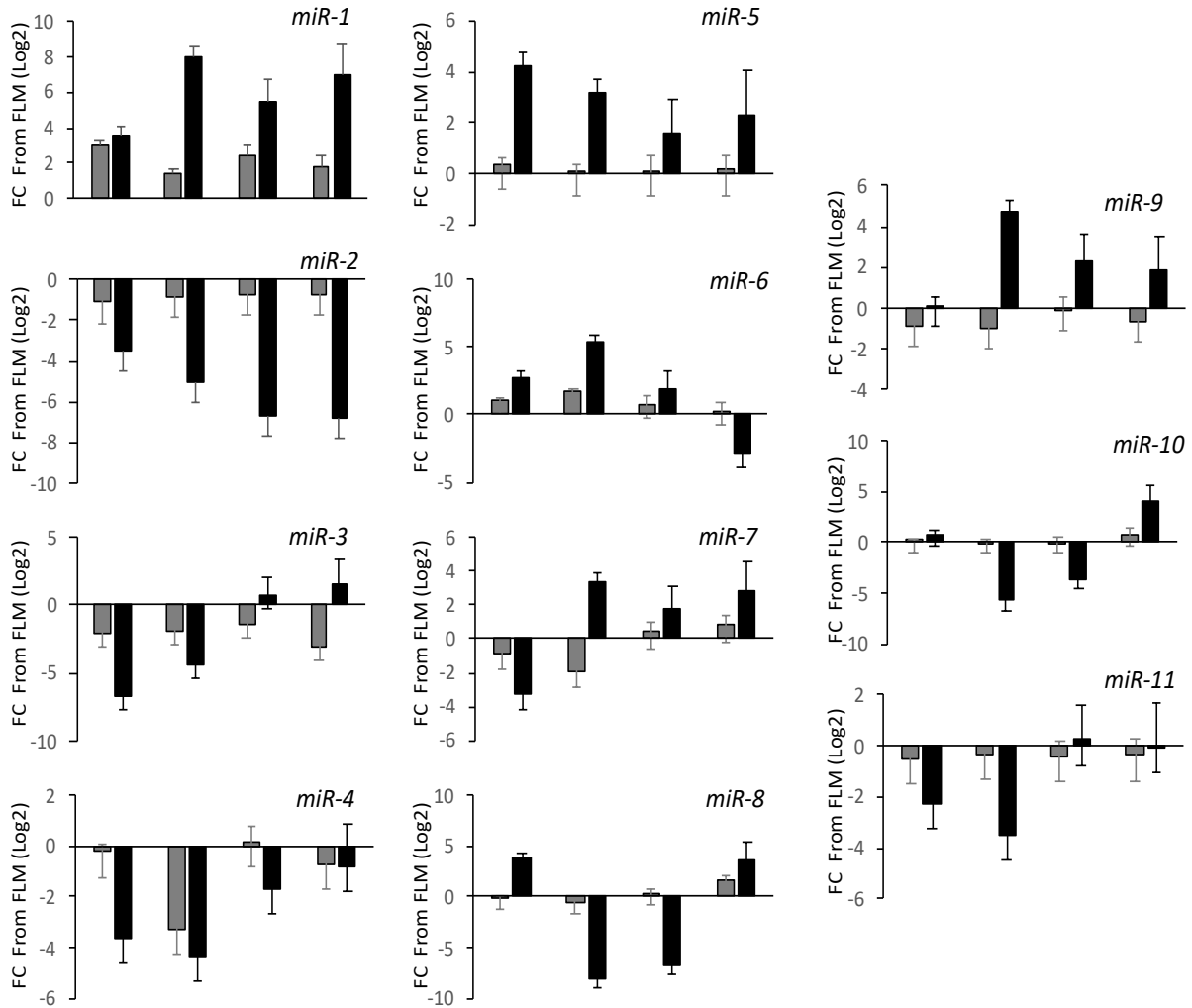


Fig. S1: Quantitative PCR verification of Illumina sequencing results. (A) Comparison of fold-change (FC) of expression patterns of *P. microcarpus* miRs in hyphae colonizing *E. grandis* root tips across four timepoints (pre-symbiosis, 24 hrs post contact, 48 hrs post contact, and 2 weeks post contact from left to right in each graph) versus axenically grown free living mycelium (FLM). These graphs compare between Illumina sequencing (dark grey bars) and quantitative PCR (black bars). \pm SE.

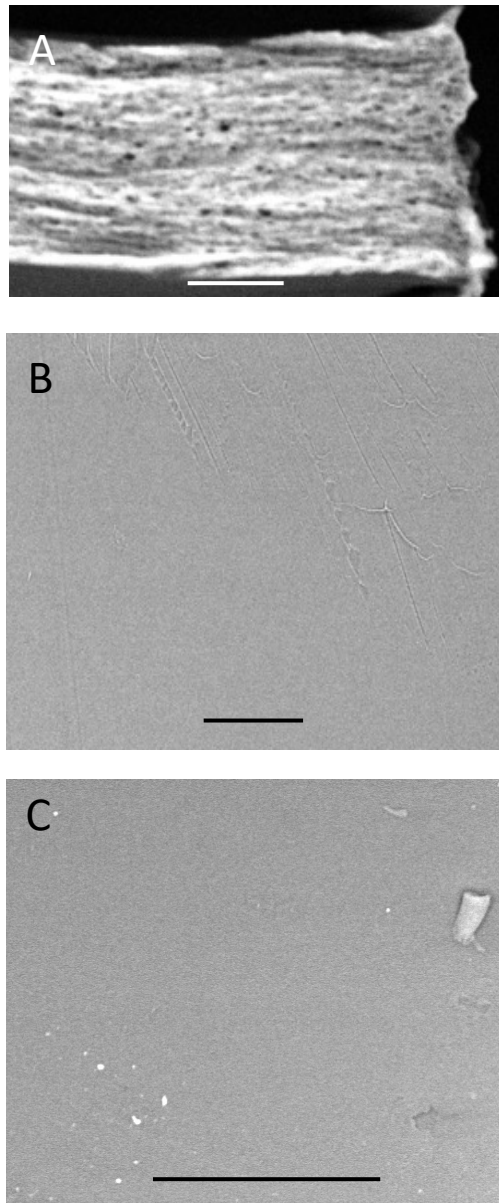


Fig. S2: Pre-symbiotic experimental verification of cellophane porosity and integrity following fungal growth. (A) View of *P. microcarpus* growing on the cellophane membrane with the membrane rolled to demonstrate that the fungal hyphae are not penetrating the membrane. Scale Bar = 7 μm **(B)** Top view of cellophane membrane upon which no fungus has grown showing the overall integrity of the material (rips and holes would appear as a dark black color). **(C)** Top view of cellophane membrane upon which *P. microcarpus* has grown for the duration of the experiment and then removed to show the continued integrity of the material. Scale bar for B-C = 50 μm .

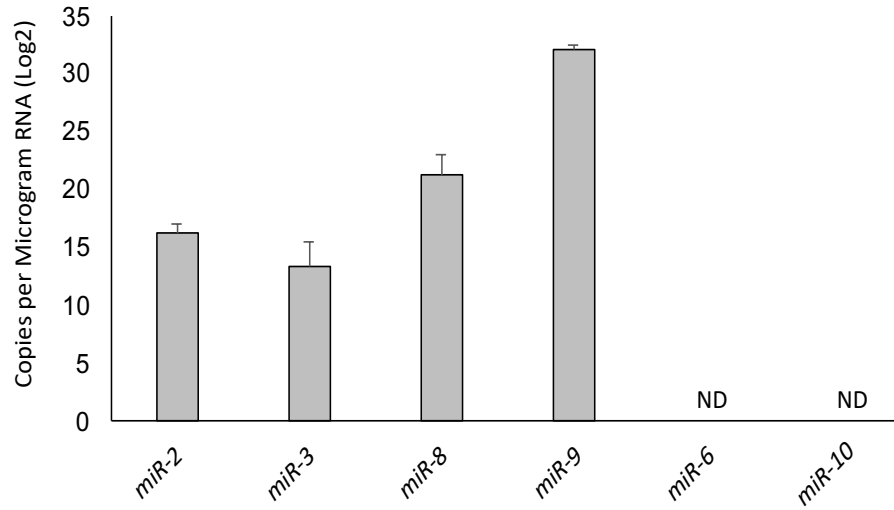


Fig. S3: Total estimated copy numbers of six *P. microcarpus* miRs in RNA extracted from *E. grandis* pre-symbiotic roots. Four of these miRNA were found in Illumina sequencing to be present in the pre-symbiotic roots (*Pmic_miR-2, 3, 8, 9*) and two were not (*Pmic_miR-6, 10*). These quantitative PCR results corroborate those initial findings. All experiments used three biological replicates. \pm SE; ND = not detected.

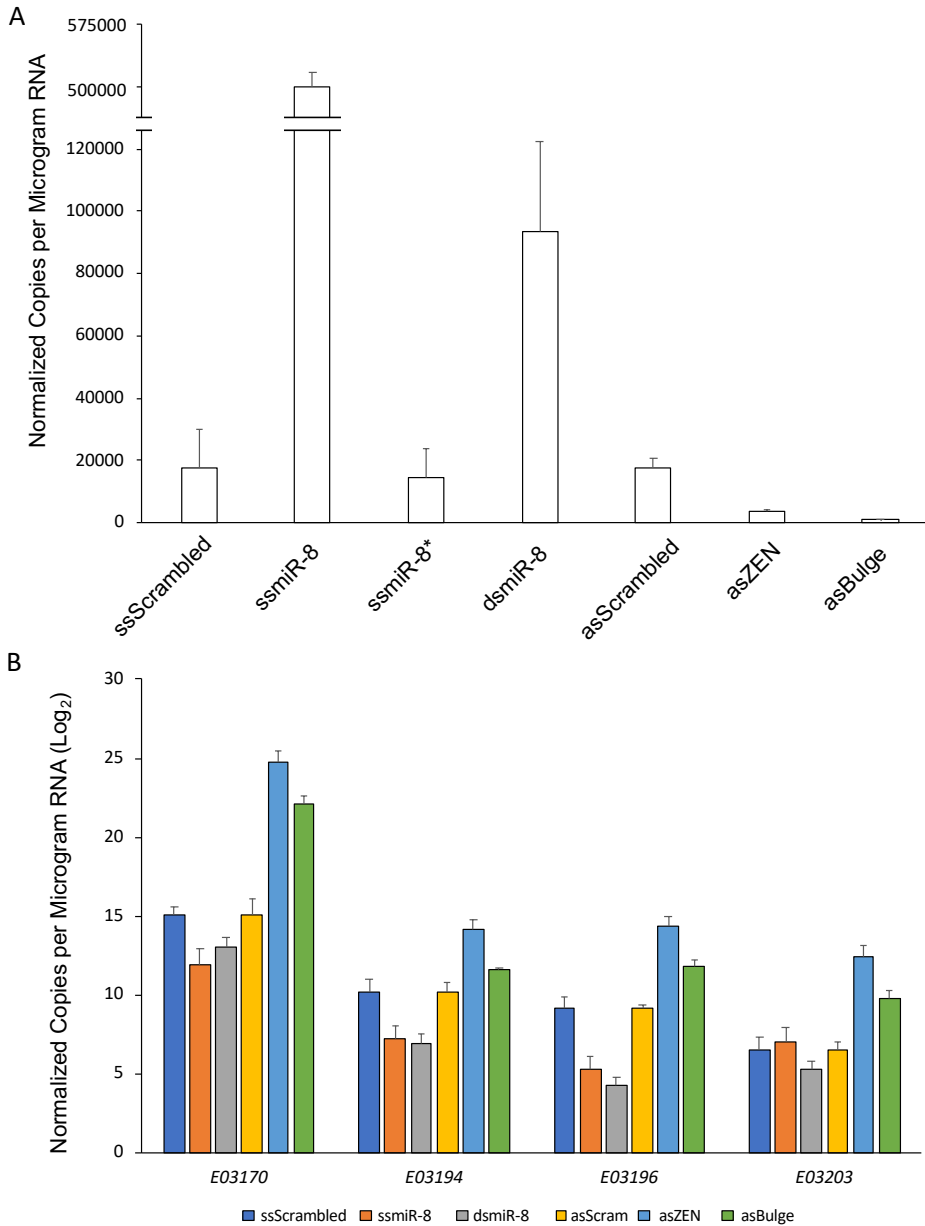


Fig. S4: Quantitative PCR analysis of normalized transcript copy number for *Pmic_miR-8* and putative *E. grandis* target genes. (A) Total estimated copy numbers of *P. microcarpus miR-8* transcripts in RNA extracted from *E. grandis* roots colonized by *P. microcarpus* and treated with mature single stranded *Pmic_miR-8* (ssmiR-8), or mature antisense *Pmic_miR-8 (ssmiR-8*), or double-stranded *Pmic_miR-8* (dsmiR-8) as compared to treatment with a scrambled miRNA (ssScrambled). We also tested *E. grandis* mycorrhizal root tips treated with either a single stranded ZEN-tagged antisense *Pmic_miR-8* inhibitor (i.e. repression; asZEN) or a single stranded antisense *Pmic_miR-8* with designed bulge mis-match at nucleotides 10-11 (asBulge) as compared to a scrambled inhibitor sequence (asScrambled). All values are the result of three biological replicates, \pm SE; (B) Total estimated copy numbers of *E. grandis* transcripts (*Eucgr.E03170*; *Eucgr.E03194*; *Eucgr.E03196*; *Eucgr.E03203*) in RNA extracted from the same tissues as used in (A). All values are the result of three biological replicates, \pm SE**

SI Table:

Table S1: Details of miRNA and LNA probes used in this study.

Nucleotide name	Description	5'/3' Modification	Oligo sequence (5'-3')
<i>ssScrambled</i>	Control for spray supplementation		CGUUAUUCGCGUAUAAUACGCGU
<i>ssmiR-8*</i>	Spray control with complementary strand		CAAGUCGAGUGGAGAAUCAA
<i>ssmiR-8</i>	Mature <i>Pmic_miR-8</i> for spray supplementation		UAUUCUCUCCUUGACUCCCC
<i>dsmiR-8</i>	Mature <i>Pmic_miR-8</i> for spray supplementation		Same as <i>ssmiR-8</i> , but as a dimer with <i>ssmiR-8*</i>
<i>asScrambled</i>	Control for spray inhibitors	ZEN at both 5' and 3' ends	G-ZEN-GUUGUCCUACCUAGGGCGC-ZEN
<i>asZEN</i>	Spray inhibitor for <i>Pmic_miR-8</i>	ZEN at both 5' and 3' ends	G-ZEN-GGAAGUCAAGGAGAGAAU-ZEN
<i>asBulge</i>	Spray inhibitor for <i>Pmic_miR-8</i> with mismatch		GGAAGUCAACUAGAGAGAAUA
anti-<i>Pmic_miR-8</i>	LNA probe used for ISH	5,6-FAM at the 5' end	AGTCAAGGAGAGAATA
Scrambled probe	LNA probe used for ISH. Sequence derived from (11).	5,6-FAM at the 5' end	GTGTAACACGTCTATACGCCA

Legends for Dataset S1 to S7:

Dataset S1: Normalized read counts of unique sequences that mapped to the *P. microcarpus* genome in the pre-symbiosis root sRNA Illumina sequencing library

Dataset S2: Mapping of the pre-symbiotic root sRNA Illumina library against the *P. microcarpus* small RNA-forming loci identified in this study.

Dataset S3: Genome coordinates of all *P. microcarpus* small RNA-forming loci identified by Shortstack (including miRNA and other small RNA)

Dataset S4: psRNATarget prediction of putative *Eucalyptus grandis* targets for all identified *P. microcarpus* miRNAs. The likelihood of a transcript being targeted by the miRNA increase as the 'expectation' value decreases. Only Expectation values <3 are reported.

Dataset S5: psRNATarget prediction of putative endogenous targets for all identified *P. microcarpus* miRNAs. The likelihood of a transcript being targeted by the miRNA increase as the 'expectation' value decreases. Only Expectation values <3 are reported.

Dataset S6: Summary of smRNA Illumina sequencing libraries used in this study

Dataset S7: Primers used in this study.

SI References:

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