Identification of MicroRNAs from *Eugenia uniflora* by High-Throughput Sequencing and Bioinformatics Analysis

Frank Guzman¹², Mauricio P. Almerão², Ana P. Körbes¹, Guilherme Loss-Morais², Rogerio Margis¹²³*

¹ PPGGBM, Departamento de Genética, Universidade Federal do Rio Grande do Sul - UFRGS, Porto Alegre, Rio Grande do Sul, Brazil, ² PPGBCM, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul - UFRGS, Porto Alegre, Rio Grande do Sul, Brazil, ³ Departamento de Biofísica, Universidade Federal do Rio Grande do Sul - UFRGS, Porto Alegre, Rio Grande do Sul, Brazil

**Abstract**

**Background:** microRNAs or miRNAs are small non-coding regulatory RNAs that play important functions in the regulation of gene expression at the post-transcriptional level by targeting mRNAs for degradation or inhibiting protein translation. *Eugenia uniflora* is a plant native to tropical America with pharmacological and ecological importance, and there have been no previous studies concerning its gene expression and regulation. To date, no miRNAs have been reported in Myrtaceae species.

**Results:** Small RNA and RNA-seq libraries were constructed to identify miRNAs and pre-miRNAs in *Eugenia uniflora*. Solexa technology was used to perform high throughput sequencing of the library, and the data obtained were analyzed using bioinformatics tools. From 14,489,131 small RNA clean reads, we obtained 1,852,722 mature miRNA sequences representing 45 conserved families that have been identified in other plant species. Further analysis using contigs assembled from RNA-seq allowed the prediction of secondary structures of 25 known and 17 novel pre-miRNAs. The expression of twenty-seven identified miRNAs was also validated using RT-PCR assays. Potential targets were predicted for the most abundant mature miRNAs in the identified pre-miRNAs based on sequence homology.

**Conclusions:** This study is the first large scale identification of miRNAs and their potential targets from a species of the Myrtaceae family without genomic sequence resources. Our study provides more information about the evolutionary conservation of the regulatory network of miRNAs in plants and highlights species-specific miRNAs.

**Citation:** Guzman F, Almerão MP, Körbes AP, Loss-Morais G, Margis R (2012) Identification of MicroRNAs from *Eugenia uniflora* by High-Throughput Sequencing and Bioinformatics Analysis. PLoS ONE 7(11): e49811. doi:10.1371/journal.pone.0049811

**Editor:** Abidur Rahman, Iwate University, Japan

**Received** June 18, 2012; **Accepted** October 17, 2012; **Published** November 15, 2012

**Copyright:** © 2012 Guzman et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This research was supported by grants from FAPERGS (Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). FG received a PhD fellowship from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). APK, MPA and RM have PNPD/CAPES, PDI/CNPq Research/CNPq fellowships, respectively. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: rogerio.margis@ufrgs.br

**Introduction**

*Eugenia uniflora* is a tropical fruit tree native to South America. The shrubby tree produces edible cherry-like fruits, which are locally known as pitanga or the Brazilian cherry. This species belongs to the Myrtaceae family, which is characterized by the presence of tannins, flavonoids, monoterpenes and sesquiterpenes whose presence and concentration varies between specimens from different geographical locations [1–3]. Extracts from pitanga leaves contain interesting biological properties that have been reported in several studies, and pitanga juice is used in folk medicine as a diuretic, antirheumatic, antipyretic, antidiarrhetic and antidiabetic [4–9]. *E. uniflora* is also an important ecological model to study because it grows in areas of medium and large levels of rainfall and can also be found in different vegetation types and ecosystems [10]. The variation in the metabolite concentration and the adaptability to different environments observed in *E. uniflora* indicating that these are the result of the transcriptional regulation of many genes involved in metabolic and signaling pathways.

MicroRNAs (miRNAs) are small non-coding regulatory RNAs widely found in unicellular and multicellular organisms that act as regulators of gene expression at the post-transcriptional level on genes containing miRNA target sites [11]. Mature miRNAs are single-stranded RNA molecules of approximately 21 nucleotides (nt) in length processed from a precursor molecule (pre-miRNA) [12]. To regulate protein-coding genes, the mature miRNA binds with perfect or imperfect complementarity to sites in the 5’ or 3’ untranslated regions (UTR) or coding sequences (CDS) of genes, which leads to mRNA degradation or translation inhibition [13–14]. In plants, miRNAs have diverse biological functions and are involved in the regulation of optimal growth and development as well as other physiological processes, including abiotic and biotic stress responses [15]. Several studies showed that many miRNAs are conserved across different plant families [16–17]. However, family- and species-specific miRNAs that are expressed in lower
levels and probably have evolved more recently have been reported [19].

In the present study, in order to evaluate the importance of miRNAs in the regulation of gene expression and metabolic pathways in *E. uniflora*, we constructed small RNA (sRNA) and poly(A) RNA-seq libraries from leaves and sequenced the libraries with high throughput Solexa technology. The sequencing data were analyzed to identify conserved and novel miRNAs and their respective targets. This work represents the first report of miRNAs identified in Myrtaceae.

**Methods**

**Plant Material and RNA Isolation**

Total RNA was isolated from *E. uniflora* leaves using the CTAB method [19]. RNA quality was evaluated by electrophoresis on a 1% agarose gel, and quantification was determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

**Deep Sequencing**

Total RNA (>10 μg) was sent to Fasteris SA (Plan-les-Ouates, Switzerland) for processing. One sRNA library was constructed and sequenced using the Illumina HiSeq2000 platform. Briefly, the construction of the sRNA library consisted of the following successive steps: acrylamide gel purification of the RNA bands corresponding to a size range of 20–30 nt; ligation of the 3p and 5p adapters to the RNA in two separate subsequent steps, each followed by acrylamide gel purification; cDNA synthesis followed by acrylamide gel purification; and a final step of PCR amplification to generate a cDNA colony template library for Illumina sequencing.

The polyadenylated transcript sequencing (RNA-seq) was performed using the following successive steps: poly-A purification; cDNA synthesis using a poly-T primer shotgun method to generate inserts of 500 nt, 3p and 5p adapter ligations; pre-amplification; colony generation; and sequencing. The Illumina output data included sequence tags of 100 bases.

**Accession Numbers**

Sequencing data are available at the NCBI Gene Expression Omnibus (GEO) ([http://www.ncbi.nlm.nih.gov/geo]). The accession number GSE38212 contains the sequence data from the RNA-seq and sRNA libraries derived from *E. uniflora* leaves.

**Data Analysis**

The overall procedure for analyzing Illumina small libraries is shown in Figure S1. All low quality reads with FASTq values below 13 were removed, and 5’ and 3’ adapter sequences were trimmed using the Genome Analyzer Pipeline (Illumina) at Fasteris SA. The remaining low quality reads with ‘n’ were removed with PrinSeq script [20]. Sequences shorter than 18 nt and larger than 25 nt were excluded from further analysis. Small RNAs derived from Viridiplantae rRNAs, tRNAs, snRNAs and snoRNAs deposited at the tRNADb [21], SILVA rRNA [22], and NONCODE v3.0 [23] databases and from Rosales mtDNA and cpDNA sequences deposited at the NCBI GenBank database ([http://ftp.ncbi.nlm.nih.gov/]) were identified by mapping with Bowtie [24].

After cleaning the data (low quality reads, adapter sequences), the RNA-seq data were assembled into contigs using the CLC Genome Workbench version 4.0.2 (CLCbio, Aarhus, Denmark) algorithm for de novo sequence assembly using the default parameters (similarity = 0.8, length fraction = 0.5, insertion/deletion cost = 3, mismatch cost = 3). In total, 170,568 contigs were assembled and used as a reference for the discovery of pre-miRNA and target sequences.

**Identification of Conserved and Novel miRNAs**

In order to determine conserved plant miRNAs, small RNA sequences were aligned with known non-redundant Magnoliophyta miRNAs deposited at miRBase (Release 18, November 2011) using Bowtie. Complete alignment of the sequences was required, and no mismatches were allowed. To search for novel miRNAs, small RNA sequences were matched against contigs obtained through *de novo* assembly of transcripts from miRNA sequences of *E. uniflora* leaves using SOAP2 [25]. The SOAP2 output was filtered with an in-house filter tool (FilterPrecursor) to identify candidate sequences as miRNA precursors using an anchoring pattern of one or two blocks of aligned small RNAs with perfect matches. The selected candidate precursors were manually inspected using the software Tablet [26] to visualize the presence of the anchoring pattern. As miRNA precursors have a characteristic hairpin structure, the next step to select candidate sequences included secondary structure analysis by RNAfold with the annotation algorithm from the UEA sRNA toolkit [27]. In addition, perfect stem-loop structures should have the miRNA sequence at one arm of the stem and a respective anti-sense sequence at the opposite arm. In the present study miRNAs were named in two different ways: (i) miR000, when corresponding to a family with two or more miRNA loci and (ii) MIR000, to design a single locus. Finally, precursor candidate sequences were checked using the BLAST algorithm from miRbase ([www.mirbase.org]).

**Validation of miRNA by RT-PCR**

In order to validate predicted miRNAs, a series of RT-PCR were performed in RNA isolated from leaves of three individuals of *E. uniflora* occurring in the Grumari native protected area in Rio de Janeiro, Brazil. Among the analyzed miRNAs seventeen corresponded to conserved miRNAs (eun-MIR156, eun-MIR159, eun-MIR160, eun-MIR166, eun-MIR167-1, eun-MIR167-2, eun-MIR167-3, eun-MIR167-4, eun-MIR395, eun-MIR396-1, eun-MIR396-2, eun-MIR397-1, eun-MIR397-2, eun-MIR482-1, eun-MIR482-2, eun-MIR530, eun-MIR827) and ten were novel miRNAs (eun-MIR001-1, eun-MIR001-2, eun-MIR004-2, eun-MIR005, eun-MIR006, eun-MIR008, eun-MIR009, eun-MIR012, eun-MIR013, eun-MIR014). The stemloop primer, used for miRNA cDNA synthesis, was designed according to Cheng et al. [28]. The forward miRNAs primers were designed based on the full mature miRNA sequences and the reverse primer was the universal reverse primer for miRNA. The RT-PCR was performed according the conditions used by Kulcheski et al. [29]. Briefly, reactions were completed in a volume of 20 μL containing 10 μL of diluted cDNA (1:100), 0.025 mM dNTP, 1X PCR Buffer, 3 mM MgCl2, 0.25 U Hot Start Taq DNA Polymerase (Promega) and 200 nM of each reverse and forward primer. Samples were analyzed in biological triplicate in a 96-well plate, and a no-template control was included. The PCR conditions were performed in an ABI 7500 Real-Time PCR System (Applied Biosystems). The PCR products were resolved on a 2% agarose gel and analyzed using Quantity One software (Bio-Rad).

**Prediction of miRNA Targets**

Previously assembled miRNA contigs were clustered using the Gene Indices Clustering Tools ([http://compbio.dfc.+/tg/software/]) [30] to reduce any sequence redundancy. The clustering output was passed to the CAP3 assembler [31] for
multiple alignment and consensus building. Contigs that did not reach the set threshold and fell into any assembly remained as a list of singletons.

The prediction of target genes for the most abundant mature miRNAs from the conserved and novel pre-miRNAs was performed by psRNATarget [32]. The program uses a 0–5 scale to indicate the complementarity between miRNA and their target, with the smaller numbers representing higher complementary and zero corresponding to a perfect complementation. Default parameters with an expectation value of 4 and E. uniflora assembled unigenes longer than 600 bp were used. Candidate RNA sequences were then annotated by assignment of putative gene descriptions based on sequence similarity with previously identified genes annotated with details deposited in the protein database of NR and the Swiss-Prot/Uniprot protein database using BLASTx implemented in blast2GO v2.3.5 software [33]. The annotation was improved by the analysis of conserved domains/families using the InterProScan tool and Gene Ontology terms as determined by the GOslim tool from blast2GO software. At the same time, the orientations of the transcripts were obtained from BLAST annotations.

Finally, to verify if the genes targeted by the identified miRNAs regulate any metabolic pathways involved in the secondary metabolites synthesis, we obtained the enzyme EC numbers for each target gene from the blast2GO annotation. These codes were uploaded to iPATH2 server [34] to generate metabolic pathway maps.

Results

E. uniflora RNA Library Sequencing

To identify conserved and novel miRNAs in E. uniflora, sRNA library was constructed from leaves and sequenced using Solexa high-throughput technology. After removing low quality sequences, those without inserts, or those with adapter contaminants or lengths outside of the 18–25 nt range, a total of 14,849,131 reads were obtained (Table 1). The number of reads with different lengths in the redundant and non-redundant sRNA datasets is shown in Figure 1 and Table S1. The most abundant sRNA species contained 21 nt, whereas the highest sequence diversity was observed in the 24-nt fraction. Approximately 6.55% of the reads matched other types of non-coding sRNAs, such as rRNAs, tRNAs, snRNAs or snoRNAs, and 9.45% matched organellar DNA (Table 2).

As there is no genome sequence available for E. uniflora, we sequenced the mRNA transcriptome of the E. uniflora leaf for use as a reference sequence in further analyses. The pooled mRNA-seq yielded 16,759,528 reads, which were imported into the CLC Genomics Workbench and de novo assembled into 170,568 contigs with an average length of 306 bp. Contigs and non-assembled reads with minimum lengths of 100 bp were further considered. The contigs ranged in size between the minimum set threshold of 100 bp and 7,808 bp (N50 = 447 bp), with 22,308 contigs more than 500 bp in length.

Identification of Conserved miRNAs in E. uniflora

There are 4,677 miRNAs from 47 Magnoliophyta species deposited in miRBase. To identify conserved miRNAs in E. uniflora, the small RNA library was matched against a set of 2,585 unique, mature plant miRNA sequences from the database. In total, 1,852,722 reads perfectly matched 204 known miRNAs (Figure 2 and Table S2). All identified sequences are distributed in 45 miRNA families, with an average of approximately 4 miRNA members per family. The largest family was miR166 with 21 members, which include isoforms found in several plant species. The miR156 (19 members), miR396 (15 members) and miR395 (14 members) families were the second, third and fourth largest miRNA families, respectively. Of the remaining miRNA families, 23 contained 2 to 10 members, and 18 were represented by a single member (Figure 2).

| Table 1. Summary of data from sequencing of E. uniflora small RNA library. |
|-------------------------|-------------------------|--------------------|
| **Type**                | **Number of reads**     | **Percentage (%)** |
| Total reads*            | 14,849,131              | 100                |
| 18–25 nt                | 12,759,506              | 86                 |
| <18 nt                  | 1,554,975               | 10                 |
| >25 nt                  | 534,650                 | 4                  |

*Reads with high quality with lengths of 1 to 44 nt.

doi:10.1371/journal.pone.0049811.t001

| Table 2. Categorization of E. uniflora noncoding and organellar small RNAs*. |
|-------------------------|-------------------------|--------------------|
| **Small RNA type**      | **Number of reads**     | **Percentage (%)** |
| miRNA                   | 1,852,722               | 14.52%            |
| tRNA                    | 765,989                 | 6.00%             |
| tRNA                    | 67,491                  | 0.53%             |
| snRNA                   | 1,555                   | 0.01%             |
| snoRNA                  | 859                     | 0.01%             |
| mtRNA                   | 159,106                 | 1.25%             |
| cpRNA                   | 1,046,305               | 8.20%             |
| Other sRNA              | 8,865,479               | 69.48%            |

*18–25 nt reads considered.

doi:10.1371/journal.pone.0049811.t002
With respect to the abundance of each miRNA family, the frequencies varied from 1 read (7 families) to 636,093 reads (miR167), indicating that expression varies significantly among different miRNA families. This relative abundance is also observed in certain members from the same family. For example, the abundance of miR167 varied from 98 to 616,862 reads, as was the case for some other families, such as miR166 (1 to 381,733 reads), miR159 (2 to 235,279 reads) and miR396 (2 to 217,485 reads). These results indicate that different members have variable expression levels within one miRNA family.

Since the genome of *E. uniflora* is not publically available, the small RNA library was matched against a set of *de novo* assembled contigs from the *E. uniflora* leaf RNA-seq to identify putative miRNA precursor sequences. Candidate sequences with hairpin-like structures and mature miRNAs anchored in either or both of the 5p or 3p arms were further considered (Figure S2). Initial analysis allowed for the identification of 25 precursor sequences grouped into 15 conserved families (Table 3). The average value of MFE was -66.51 in these precursors and included two precursors (MIR167-2 and MIR169) with extreme values due to their long sizes. With respect to the % GC and MFEI, the average values were 47.63 and -0.94, respectively.

Within the identified families, MIIR167 was the most abundant, with 676,895 reads, and contained 4 members (MIIR167-1, MIIR167-2, MIIR167-3, and MIIR167-4). In addition, several miRNA isoforms were detected in the libraries, and some of these were more abundant than the known miRNAs reported in miRBase for other plants (Figure 3). Furthermore, in the family MIIR397, one precursor was identified with a typical structure and mature reads in the sense and antisense orientations. Both orientations were considered two independent precursors from the same family for the following analysis.

**Identification of Novel miRNAs in *E. uniflora***

Using the previously described criteria in the identification of conserved pre-miRNAs, we obtained another 17 potential miRNA candidates grouped into 15 families (Table 4). In addition to the hairpin structure, the detection of miRNA* in 14 precursors is a strong indication to consider these miRNAs as true candidates. Comparisons among the mature sequences of candidate miRNAs and those miRNAs deposited in miRBase suggest that these candidates are novel miRNAs that have not been identified in others species and are possibly specific to the Myrtaceae family. These novel miRNAs displayed an average negative folding value of -137.89, which included 4 miRNAs with long sizes similarly observed in some previously identified conserved pre-miRNAs. With respect to the % GC and MFEI, the average values were 42.86 and -1.05, respectively. In addition, one novel pre-miRNA was found with mature sequences in the sense and antisense orientations and was considered to represent 2 members of the same family (nMIIR001-1 and -2).

**Biological Confirmation of Identified miRNAs in *E. uniflora***

The stem-loop RT-PCR method was used to validate the expression of seventeen conserved miRNAs (eun-MIIR156, eun-MIIR159, eun-MIIR160, eun-MIIR166, eun-MIIR167-1, eun-MIIR167-2, eun-MIIR167-3, eun-MIIR167-4, eun-MIIR395, eun-MIIR396-1, eun-MIIR396-2, eun-MIIR397-1, eun-MIIR397-2, eun-MIIR402-1, eun-MIIR402-2, eun-MIIR530, eun-MIR327) and ten novel miRNAs (eun-MIIR001-1, eun-MIIR001-2, eun-MIR004-2, eun-MIIR005, eun-MIIR006, eun-MIIR008, eun-MIIR009, eun-MIIR012, eun-MIIR013, eun-MIIR014). We confirmed that these miRNAs were expressed in three different individuals collected *in situ* (Figure S3).

**Identification and Classification of miRNA Targets**

To understand the biological function of miRNAs in *E. uniflora*, the putative miRNA target sites of miRNA candidates were identified by aligning the most abundant mature miRNAs of each conserved and novel precursor to a set of *E. uniflora* assembled unigenes using psRNA target with default parameters and a maximum expectation value of 4. We found 87 potential targets in total, where 52 were targets of conserved miRNAs and 35 were targets of novel miRNAs, with an approximate average of 3 targets per miRNA. Detailed annotation results are given in Table 5 and S3.

Among the most important miRNA targets, also previously identified in other plants, we found the squamosa promoter binding protein (SBP)-like (SPL) genes, which are targets of the miR156 family and have functions that are conserved across plant species [17], affecting diverse developmental processes, such as leaf development, shoot maturation, phase change and flowering in plants [33-40]. We also identified the auxin response factor (ARF), a plant-specific family of DNA binding proteins involved in...
### Table 3. Pre-miRNAs identified in *E. uniflora* with sequence similarities to plant conserved miRNA families.

| miRNA   | Precursor miRNA | Mature miRNA | 5p sequence more abundant | Read count | 3p more abundant sequence | Read count | Total reads |
|---------|-----------------|--------------|---------------------------|------------|---------------------------|------------|-------------|
| eun-MIR156 | Contig92889 97 54.64 | -55.20 | TGAGCAAGAGATAGAGACAC | 83933 | GCTCTCCCTCTTGACACA | 1 | 85396 |
| eun-MIR159 | Contig93245 163 45.40 | -34.27 | AGCTCTGGTCTATGAGGAC | 376 | CTTGCATATGCAAGGAGCC | 493 | 1302 |
| eun-MIR160 | Contig81816 116 53.45 | -56.91 | TGCGCTGGCTCTGGATGCA | 293 | GCGGTAGAGGAGCGAAGCATA | 22 | 323 |
| eun-MIR162 | Contig165400 108 48.15 | -33.15 | GGAGGCAGCGGTTCATGGATC | 24 | TGATAAAAATGCTGCAGTCCA | 10713 | 10884 |
| eun-MIR166 | Contig94223 228 43.42 | -0.73 | GGAATGTTGTCTGGCTCGAGG | 11016 | TCGGACCAGGCTTCATTCCC | 381733 | 409546 |
| eun-MIR167-1 | Contig26350 90 45.56 | -0.75 | AGCTGCTGGTCTATGAGGAC | 376 | CTTGCATATGCAAGGAGCC | 493 | 1302 |
| eun-MIR167-2 | Contig163487 615 37.40 | -26.60 | TGAGCTGGCCAGCAGTCCTGG | 32188 | TCGGACCAGGCTTCATTCCC | 381733 | 409546 |
| eun-MIR167-3 | Contig784s 81 48.15 | -0.99 | TGAAGCTGCCAGCATGATCTCA | 16305 | ATCAGATCATGTGGCAGCTTCACC | 73 | 22056 |
| eun-MIR167-4 | Contig784a 81 48.15 | -0.99 | TGAAGCTGCCAGCATGATCTCA | 16305 | ATCAGATCATGTGGCAGCTTCACC | 73 | 22056 |
| eun-MIR169 | Contig142088 730 39.18 | -22.03 | TTATAGGCGATTGGAGGTATG | 876 | TTAGCTAAAGTCGGTCCCA | 6818 | 8961 |
| eun-MIR172-1 | Contig13567 92 42.39 | -1.02 | GCAGCATCATCAAGATTCACA | 12 | AGAATCTTGATGCTGCAT | 495 | 1076 |
| eun-MIR172-2 | Contig85928 165 43.64 | -0.99 | GTAGCATCTAGACTTGCCACA | 33 | AGAATCTTGATGCTGCAT | 495 | 1076 |
| eun-MIR172-3 | Contig114717 88 51.14 | -1.06 | GTGAGCATCATCAAGATTCACA | 12 | AGAATCTTGATGCTGCAT | 495 | 1076 |
| eun-MIR395 | Contig94388 160 42.50 | -1.01 | TTCCAGCAGCTTCTAAGCTC | 217485 | GTTCAATAAAGCTGTGGGAAG | 2028 | 223828 |
| eun-MIR396-1 | Contig87345s 126 51.59 | -0.91 | TCCACAGCTTCTAACGACTC | 23061 | GTCCAGATCTGTGCTGGAAG | 12981 | 64439 |
| eun-MIR397-1 | Contig87345a 126 51.59 | -0.91 | TCCACAGCTTCTAACGACTC | 23061 | GTCCAGATCTGTGCTGGAAG | 12981 | 64439 |
| eun-MIR482-1 | Contig88445 153 53.90 | -1.14 | GAGGCAGCTTGCAACGAT | 20 | TCAAGCGTCACTGCTCAATGAT | 273 | 322 |
| eun-MIR482-2 | Contig88445 153 53.90 | -1.14 | GAGGCAGCTTGCAACGAT | 20 | TCAAGCGTCACTGCTCAATGAT | 273 | 322 |
| eun-MIR482-3 | Contig85065 169 50.89 | -0.88 | TCTGATTCTGCAGTCCACC | 185 | AGTGCAGGGTCGAGAATGG | 12 | 280 |
| eun-MIR530 | Contig153308 128 42.19 | -0.91 | TCCACAGCTTCTAACGACTC | 23061 | GTCCAGATCTGTGCTGGAAG | 12981 | 64439 |
| eun-MIR535-1 | Contig87345s 126 51.59 | -0.91 | TCCACAGCTTCTAACGACTC | 23061 | GTCCAGATCTGTGCTGGAAG | 12981 | 64439 |
| eun-MIR535-2 | Contig87345a 126 51.59 | -0.91 | TCCACAGCTTCTAACGACTC | 23061 | GTCCAGATCTGTGCTGGAAG | 12981 | 64439 |
| eun-MIR535-3 | Contig71803 100 49.00 | -0.96 | TGACACGGAGAGAGACACC | 62562 | GTGCCTCCTCTCGTTCACA | 4199 | 76334 |
| eun-MIR535-4 | Contig93928 81 45.68 | -1.20 | TCTTGGTACAGGCCGTACAT | 27 | TTAGATGACAACTAGCAAGACA | 266 | 304 |

MFE: minimal folding free energy (kcal/mol); AMFE: Adjusted MFE; MFEI: minimal folding free energy index; ND: not detected.
hormone signal transduction that are targets for the miRNA families miR167 and miR160 [15,41,42]. Another important gene identified and targeted by miR162, with a significant role in the regulation of gene expression, is the pentatricopeptide repeat gene (PPR). This gene belongs to a large family implicated in post-transcriptional processes, such as splicing, editing, processing and translation specifically in organelles like mitochondria and chloroplasts [43]. These results substantiate the in silico identification of conserved and novel targets from *E. uniflora*.

All targets regulated by the conserved and novel miRNAs identified in this study were subjected to GO analysis to evaluate their potential functions. The categorization of these genes, according to biological processes, cellular components and molecular functions, is summarized in Figure 4. Based on biological processes, these targets were classified into 13 categories, and the three most overrepresented GO terms, either for conserved or novel miRNAs, were cellular processes, metabolic processes and responses to stimulus, suggesting that *Eugenia* miRNAs are involved in a broad range of physiological functions. Categories based on molecular function revealed that the target genes were related to 7 functions, and the four most frequent terms were protein binding, nucleotide binding, hydrolase activity and nucleic acid binding. In the category of cellular components, the analysis revealed that the protein products from the genes targeted by conserved and novel miRNAs are expressed mainly in the plastid and nucleus.

The iPATH2 server was used to produce an overview of the metabolic pathways involved in the secondary metabolites synthesis and potentially regulated by miRNAs in *E. uniflora*. Our results showed that three enzymes involved in several types of metabolism and secondary metabolites are regulated by identified miRNAs (Figure S4). The phosphoglycerate mutase is a potential target of eun-MIR396-2 and is involved in the pathway of gluconeogenesis while the hydroxyphenylpyruvate reductase is
Table 4. New putative miRNA precursors identified in *E. uniflora*.

| miRNA     | Precursor miRNA | Mature miRNA | 5p more abundant sequence | Read count | 3p more abundant sequence | Read count | Total reads |
|-----------|-----------------|--------------|---------------------------|------------|---------------------------|------------|-------------|
| eun-nMIR001-1 | Contig164780s 820 54.39 | –451.70 | –55.09 | –1.01 | TCGGCCTGCAATTTCGATT | 185919 | ATCCAGAATTGGCAGCCGTT | 110 | 192103 |
| eun-nMIR001-2 | Contig164780a 820 54.39 | –446.00 | –54.39 | –1.00 | CAAATTCTGGATTCATG | 20 | TCGAATCTGAATCCAGAATT | 2 | 63 |
| eun-nMIR002 | Contig29785 173 30.06 | –56.70 | –32.77 | –1.09 | CGAAATGATGTTGGTTGATGCT | 18 | CGATCTAATCATATTTCGCC | 2 | 21 |
| eun-nMIR003 | Contig121100 110 48.18 | –63.20 | –57.45 | –1.19 | TACTGGTCCGTGATTCATC | 88 | TGGATCAATAGAACGAGCAGGTGA | 157 | 561 |
| eun-nMIR004-1 | Contig37387s 104 29.81 | –47.10 | –45.29 | –1.52 | TCGAATCCACTATATCTCTC | 3 | TAGATATGCTGATTCGAT | 9 | 13 |
| eun-nMIR004-2 | Contig37387a 104 29.81 | –46.40 | –44.62 | –1.50 | ND | ND | ND | 12 | 30 |
| eun-nMIR005 | Contig143563 106 34.91 | –21.40 | –20.19 | –0.58 | ND | ND | –GAGATCTGATTCGTTAATTGGA | 314 | 385 |
| eun-nMIR006 | Contig113617 113 33.63 | –42.70 | –37.79 | –1.12 | TCTCTGTGAGATCTGAAATA | 19 | TTTGTCCGGATGAAACAGGAAT | 18 | 55 |
| eun-nMIR007 | Contig116664r 96 46.88 | –55.80 | –58.13 | –1.24 | TAGGTGCTAGATCGCTATTTAG | 211 | TAAATGGTGAATCGTACTTAA | 4464 | 5750 |
| eun-nMIR008 | Contig79716 426 43.66 | –228.00 | –53.52 | –1.23 | TCGAGGCCTCCCAAGATTTG | 313 | ATCTGTGGAAGAATCCTGAT | 8403 | 13617 |
| eun-nMIR009 | Contig81320 1545 37.41 | –397.90 | –25.75 | –0.69 | TTAAGCTCAGGCCATTCTCGACT | 5774 | TGGAGGTGTGGCTGCTGAGCT | 1968 | 11577 |
| eun-nMIR010 | Contig24248 350 48.86 | –143.30 | –40.94 | –0.84 | TGCCTGGTTCGCTTCCCAAT | 309 | TCCTGGAACAAAGATGCTGAT | 6340 | 10307 |
| eun-nMIR011 | Contig164559 207 51.21 | –98.70 | –47.68 | –0.93 | GCTCGAGGCTAGTTGCGCC | 1553 | CGGAACTCGGAGCTTCCAGATC | 110 | 2884 |
| eun-nMIR012 | Contig167957 164 35.98 | –91.80 | –55.98 | –1.56 | CATGGTACAGTTGCGTCA | 126 | TCTGGACAGCCGACCTTATT | 3 | 243 |
| eun-nMIR013 | Contig87665 179 56.42 | –84.90 | –47.43 | –0.84 | TGAACGACATCAAGACCGAG | 155 | TCTGCTCCGGTCTTACCTGAA | 2 | 172 |
| eun-nMIR014 | Contig200629r 87 55.17 | –23.40 | –26.90 | –0.49 | ND | –GACATCTAGCCTTACCTGCTG | 30 | 159 |
| eun-nMIR015 | Contig165886 124 37.90 | –45.10 | –36.37 | –0.96 | ND | –CAATGAAAAGCTTTGCAAGTTG | 21 | 22 |

MFE: minimal folding free energy (kcal/mol); AMFE: Adjusted MFE; MFEI: minimal folding free energy index; ND: no detected.

doi:10.1371/journal.pone.0049811.t004
targeted by eun-MIR162 and is involved in terpenoid-quinone, tropane, piperidine and pyridine biosynthesis. In a similar way, eun-nMIR007 regulates primary-amine oxidase, an enzyme involved in the tropane, piperidine, pyridine and isoquinoline alkaloid biosynthesis.

**Discussion**

Though several miRNAs have been identified via computational or experimental approaches in different plant families, there is no sequence or functional information available about miRNAs in any Myrtaceae species, which are economically important in the spice, fruit, timber and pharmacology industries [44].

We used Solexa technology for deep sequencing of a small RNA library to identify miRNAs in *E. uniflora*. The length distribution pattern obtained indicates that the majority of the redundant small RNAs from the library were 21 nt in length, which is atypical because 24 nt is the most abundant size produced by DCL3 in other plants [45]. This distribution pattern is similar to those observed in previous reports of plant small RNA sequencing using Solexa technology, such as wheat [46], grapevine [47], melon [48] and trifoliate orange [49], suggesting that the composition of the small RNA population varies among species. Additionally, other important causes for this variation include the developmental stage and environmental conditions in which the sample was collected. Contrary to the results observed with the redundant sequences, the analysis of the unique sequences showed that 24 nt was the

---

**Table 5. Predicted targets of novel miRNAs in *E. uniflora*.**

| miRNA          | Inhibition     | Score* | Putative Function                                   |
|----------------|---------------|--------|----------------------------------------------------|
| eun-nMIR001    | Cleavage 1.5  | Atp-dependent helicase rhp16-like           |
|                | Cleavage 3    | Cytochrome p450                             |
|                | Cleavage 3.5  | Long chain acyl- synthetase 9             |
|                | Cleavage 3.5  | Kinesin-related protein                     |
|                | Translation 3 | Transcription initiation factor iiB        |
|                | Translation 3 | Brassinazole-resistant 1                   |
|                | Translation 3.5 | Myosin family protein with dill domain     |
| eun-nMIR002    | Cleavage 3    | Serine threonine-protein phosphatase 2a regulatory subunit b subunit alpha-like |
|                | Cleavage 3.5  | Probable receptor-like protein kinase at1g57000-like |
| eun-nMIR003    | Cleavage 4    | Udpglycosyltransferase 74b1                |
| eun-nMIR004    | Cleavage 2.5  | Auxin efflux carrier protein               |
|                | Cleavage 3.5  | Sucrose nonfermenting 4-like               |
|                | Translation 3.5 | Type i inositol- -trisphosphate 5-phosphatase 2-like |
| eun-nMIR005    | Translation 3 | Pentatricopeptide repeat-containing protein |
|                | Cleavage 3.5  | Protein reticulata-related 1               |
|                | Cleavage 3.5  | Agenet domain-containing protein            |
| eun-nMIR006    | Translation 3.5 | Outward rectifying potassium channel       |
| eun-nMIR007    | Cleavage 3.5  | Aspartate semialdehyde                     |
|                | Cleavage 3    | Primary-amine oxidase                      |
| eun-nMIR008    | Translation 3.5 | Adenosine deaminase                        |
| eun-nMIR009    | Cleavage 3    | Cc-nbs-lrr resistance protein              |
| eun-nMIR010    | Translation 4 | Ptmtpc1                                    |
|                | Cleavage 4    | Nbs-lrr resistance protein                 |
|                | Translation 4 | Cullin-1-like isoform 1                    |
| eun-nMIR011    | Translation 3 | E3 ubiquitin-protein ligase upl7           |
|                | Cleavage 3.5  | Eukaryotic peptide chain release factor subunit 1-1 |
| eun-nMIR012    | Translation 3.5 | Tho complex subunit 2                     |
| eun-nMIR013    | Translation 3.5 | Beta-amyrase                               |
| eun-nMIR014    | Cleavage 0    | Ycf68 protein                              |
| eun-nMIR015    | Cleavage 3.5  | Rna-binding motif x-linked 2               |
|                | Cleavage 3.5  | Transducin wd-40 repeat-containing protein |
|                | Cleavage 3.5  | Clp-associating protein                    |
|                | Cleavage 3.5  | Probable exocyst complex component 4-like  |
|                | Cleavage 3.5  | Photosystem i p700 apoprotein a1           |

*psRNATarget value.

doi:10.1371/journal.pone.0049811.t005
dominant read length in comparison to all other sequence lengths, and similar results have been observed in other studies [50-53]. Small RNAs of 24 nt in length are known to be involved in heterochromatin transcriptional silencing in genomes with a high content of repetitive sequences [54], indicating the possible genome complexity of E. uniflora.

In this study, we compared our small RNA library from E. uniflora against known plant miRNAs from the miRBase database and identified 204 conserved miRNAs from different species grouped into 45 families. High throughput sequencing, which has the ability to generate millions of small RNA sequences, is a powerful tool to estimate expression profiles of miRNA. This technology provides the resources to determine the abundance of various miRNA families and even distinguish among different members of a given family. In our case, we found significant differences among the number and abundance of the members identified in each family, which is in agreement with previous studies [48,55,56] and suggests that this wide variation is due to a functional divergence in the conserved miRNA families.

Although conserved miRNAs have been identified by sequencing and comparison against miRNAs from other species, most plant species-specific miRNAs remain unidentified due to their lower levels of expression, which result in a small number of sequenced reads in comparison to the conserved miRNAs [57]. For this reason, we used a new approach to identify novel miRNAs in species where genomic data and resources were not available. We made use of simultaneous sequence comparison of small RNA and RNAseq libraries. Using this methodology, we identified 17 potential miRNA candidates specific for E. uniflora. From these, 14 contained complementary antisense miRNA, which provided
more evidence for their existence as novel miRNAs, as observed in cucumber [51] and grapevine [53]. The other miRNAs that do not satisfy this last criterion require further investigation for their confirmation as miRNAs.

To understand the function of the identified miRNAs, their putative targets were predicted using a bioinformatics approach. Several identified targets of conserved miRNAs of E. uniflora are transcriptional factors, similar to the results reported in other studies [47,48,50,59]. In the case of the novel miRNA targets, we found that the transcription initiation factor ii b and the pentatricopeptide repeat-containing proteins are targeted by eun-nMIR001 and eun-nMIR005, respectively.

It has been reported in Arabidopsis that regulation of ARF17 by miR160 is important for growth and development [60], regulation of ARF6 and b by miR167 is important for development of anthers and ovules [61] and regulation of ARF10 and 16 by miR160 plays a role in root cap formation [62]. In the present study, we found that ARF17 is regulated by eun-MIR160 while other members of ARF family were not targeted by eun-MIR167. This discrepancy agrees with the previously reported in Arabidopsis because we used a leaf transcriptome as reference for the target identification. We confirm this observation not found homologs for ARF6, 8, 10 and 16 by BLASTx in E. uniflora transcriptome.

In addition, with the analysis of GO terms, we identified 3 candidate targets likely involved in the response to abiotic stress: ATP-dependent helicase rhp16-like (eun-nMIR002), sucrose nonfermenting 4-like (eun-MIR004) and serine threonine-protein phosphatase 2a regulatory subunit b” subunit alpha-like (eun-nMIR002). The sucrose nonfermenting 4-like (SNF4) protein is a subunit of the probable trimeric SNF1-related protein kinase (SnRK) complex, which may play a role in a signal transduction cascade regulating gene expression and carbohydrate metabolism in higher plants [63]. Otherwise, the serine-threonine-protein phosphatase 2A regulatory subunit b” subunit alpha-like PP2Ab” is a structural subunit of the Ser/Thr phosphatases holoenzyme (PP2A) and recent studies suggesting the possible physiological role of PP2A in the drought stress response [64]. These results indicated that the targets from novel miRNAs identified here are possibly related to the adaptation of E. uniflora to different types of stress and environmental conditions observed in nature. Future experimental validation will determine how many of these predicted targets are genuinely targeted by miRNAs in specific environmental and physiological conditions.

Interestingly, we found three miRNAs involved in the regulation of enzymes that play critical roles in secondary metabolites synthesis. These findings suggest that variation in the levels of expression of these miRNAs could alter the levels of production of certain types of secondary metabolites. It is consistent with the previous reports that the concentration of these metabolites varies between specimens of E. uniflora from different geographical locations [2,3]. More studies are necessary to confirm these preliminary findings and evaluate the correlation between the miRNA expression and secondary metabolite production.

Conclusions

In summary, this study provides the first view of the diversity of miRNAs and their abundance in Myrtaceae and strongly supports the idea that miRNAs play an important conserved role in several physiological processes, as previously proposed for other plants. Our bioinformatics analysis indicates that miRNAs might contribute to different processes by affecting multiple target genes and different signaling pathways. Although the exact function of these miRNA target genes remains to be confirmed, we believe the present study provides novel insights into the molecular processes involved in conserved miRNA function.

Supporting Information

Figure S1 Flow chart of procedures for miRNA identification. (TIFF)

Figure S2 Represenation of the predicted secondary structures of the conserved and novel miRNA precursors of E. uniflora and the locations of the more abundant mature miRNAs. (PDF)

Figure S3 Detection of miRNA expression in different E. uniflora individuals by RT-PCR. Products generated by stem-loop RT-PCR were resolved on a 2% agarose. Leaf samples from three independent Eugenia uniflora trees were used to evaluate the presence of each miRNA. (TIFF)

Figure S4 iPath secondary metabolite map showing the different pathways where are involved each evaluated enzyme. Each grey dot represents a metabolite and each colored line represents the different route affected by the enzyme targeted. In red: phosphoglycerate mutase (regulated by eun-MIR396-2). In blue: primary-amine oxidase (regulated by eun-MIR007). (TIF)

Table S1 Length distribution and read sequence abundance in the E. uniflora small RNA library. (XLS)

Table S2 Abundance of predicted plant conserved miRNAs in the small RNA library of E. uniflora. (XLS)

Table S3 Predicted transcript targets of plant conserved miRNAs in E. uniflora. (XLS)

Acknowledgments

The authors would like to thank Maria Elena Gonzalez for reviewing of the manuscript.

Author Contributions

Conceived and designed the experiments: RM. Performed the experiments: FG MPA APK GLM RM. Analyzed the data: FG MPA APK GLM RM. Contributed reagents/materials/analysis tools: FG MPA APK GLM RM. Wrote the paper: FG. Reviewed the manuscript: MPA APK GLM RM.

References

1. Burt S (2004) Essential oils: their antibacterial properties and potential applications in foods—a review. International journal of food microbiology 94: 223–253.

2. Lago JHG, Souza ED, Mariane B, Pascon R, Vallim M a, et al. (2011) Chemical and biological evaluation of essential oils from two species of Myrtaceae - Eugenia uniflora L. and Plinia transculata (O. Berg) Kausel. Molecules 16: 9827–9837.

3. Victoria FN, Lenardato EJ, Savenga L, Perin G, Jacob RG, et al. (2012) Essential oil of the leaves of Eugenia uniflora L.: antioxidant and antimicrobial properties. Food and chemical toxicology 50: 2668–2674.

4. Arai I, Amagaya S, Komatsu Y, Okada M, Hayashi T, et al. (1999) Improving the idea that miRNAs play an important conserved role in several physiological processes, as previously proposed for other plants. Our bioinformatics analysis indicates that miRNAs might contribute to different processes by affecting multiple target genes and different signaling pathways. Although the exact function of these miRNA target genes remains to be confirmed, we believe the present study provides novel insights into the molecular processes involved in conserved miRNA function.

Supporting Information

Figure S1 Flow chart of procedures for miRNA identification. (TIFF)

Figure S2 Representation of the predicted secondary structures of the conserved and novel miRNA precursors of E. uniflora and the locations of the more abundant mature miRNAs. (PDF)

Figure S3 Detection of miRNA expression in different E. uniflora individuals by RT-PCR. Products generated by stem-loop RT-PCR were resolved on a 2% agarose. Leaf samples from three independent Eugenia uniflora trees were used to evaluate the presence of each miRNA. (TIFF)

Figure S4 iPath secondary metabolite map showing the different pathways where are involved each evaluated enzyme. Each grey dot represents a metabolite and each colored line represents the different route affected by the enzyme targeted. In red: phosphoglycerate mutase (regulated by eun-MIR396-2). In blue: primary-amine oxidase (regulated by eun-MIR007). (TIFF)

Table S1 Length distribution and read sequence abundance in the E. uniflora small RNA library. (XLS)

Table S2 Abundance of predicted plant conserved miRNAs in the small RNA library of E. uniflora. (XLS)

Table S3 Predicted transcript targets of plant conserved miRNAs in E. uniflora. (XLS)

Acknowledgments

The authors would like to thank Maria Elena Gonzalez for reviewing of the manuscript.

Author Contributions

Conceived and designed the experiments: RM. Performed the experiments: FG MPA APK GLM RM. Analyzed the data: FG MPA APK GLM RM. Contributed reagents/materials/analysis tools: FG MPA APK GLM RM. Wrote the paper: FG. Reviewed the manuscript: MPA APK GLM RM.
13. Bushati N, Cohen SM (2007) MicroRNA functions. Annual Review of Cell and Developmental Biology 23: 173–205.

26. Milne I, Bayer M, Cardle L, Shaw P, Stephen G, et al. (2010) Tablet–next generation sequence assembly visualization. Bioinformatics 26: 401–402.

28. Chen C, Ridzon D a, Broomer AJ, Zhou Z, Lee DH, et al. (2005) Real-time quantitative PCR of microRNAs by stem-loop RT-PCR: Nucleic acids research 33: 2252–2253.

29. Kulcheski FR, de Oliveira LF, Molina LG, Almera˜o MP, Rodrigues F, et al. (2005) Studying the effects of oil composition, antibacterial and cytotoxicity of Eugenia uniflora L. International Journal of Aromatherapy 15: 147–152.

36. Wu G, Park MY, Conway SR, Wang J-W, Weigel D, et al. (2009) The development of Arabidopsis thaliana genetic reference data. Nucleic Acids Research 37: D159–62.

41. Wu M-F, Tian Q, Reed JW (2006) Arabidopsis microRNA167 controls patterns of ARF6 and ARF8 expression, and regulates both female and male reproductive development. Development 133: 4211–4218.

42. Drui B, Top J, Plasterk RH a, Ketting RF, Hannon GJ (2004) Processing of primary microRNAs by the Microprocessor complex. Nature 432: 231–235.

44. Okoh-Esene Rosemary, Husseini Suleiman AT (2011) Proximate and phytochemical analysis of leaf, stem and root of Eugenia uniflora (Surinam or Pitanga cherry). Journal of Natural Product and Plant Resources 1: 1–4.

45. Poethig RS (2009) Small RNAs and developmental timing in plants. Current Opinion in Plant Biology 12: 439–447.

46. Matsumura T, Kasai M, Hayashi T, Arisawa M, Momose Y, et al. (2000) Discovery of microRNA genes by inverted duplication of target gene sequences in rice. Plant Journal 23: 115–122.

47. González-Beato D, Blanco J, Donaire L, Salafí M, Mascarill-Creen A, et al. (2011) Analysis of the melanin (Cannabis indica) gene microRNAs through high-throughput pyrosequencing. BMC Genomics 12: 393.

48. Sunkar R, Li Y-F, Jagadeeswaran G (2012) Functions of microRNAs in plant reproduction. Development 139: 3251–3258.

49. Zhang J-Z, Ai X-Y, Guo W-W, Peng S-A, Deng X-X, et al. (2011) Identification of miRNAs and Their Target Genes Using Deep Sequencing and Degradation Analysis in Trifoliate Orange (Poncirus trifoliata [L.] Raf]. Molecular Biotechnology. 44: 47–51.

50. Song C, Wang G, Zhang C, Keir N, Yu H, et al. (2010) High-throughput sequencing of novel and conserved microRNAs in trifoliate orange (Citrus trifoliata). BMC Genomics 11: 431.

51. Martinez G, Forment J, Llave C, Pallais V, Gómez G (2011) High-throughput sequencing, characterization and discovery of novel and conserved cucumber miRNAs. PloS One 6: e19352.

52. Schreiber AW, Shi B-J, Huang C-Y, Langridge P, Baumann U (2011) Discovery of barley miRNAs through deep sequencing of short reads. BMC Genomics 12: 129.

53. Wang C, Wang X, Kibet NK, Song C, Zhang C, et al. (2011) Deep sequencing of grapevine flower and berry short RNA library for discovery of novel grapevine miRNAs and validation of precise sequences of grapevine miRNAs deposited in miRBase. Physiological and Molecular Plant Biology 145: 81–84.

54. Simon S, Meyers BC (2011) Small RNA-mediated epigenetic modifications in plants. Current Opinion in Plant Biology 14: 148–155.

55. Zhao C-Z, Xia H, Frazier TP, Yao Y-V, Bi Y-P, et al. (2010) Deep sequencing identifies novel and conserved microRNAs in peanuts (Arachis hypogaea L.). BMC Plant Biology 10: 3.

56. Puzey JR, Karger A, Axell K, Kramer EM (2012) Deep Annotation of Populus tremula microRNAs from Diverse Tissue Sets. PloS One 7: e30354.

57. Falahri N, Howell MD, Kaschau KD, Chapman J, Siddiqui CM, et al. (2007) High-throughput sequencing of Arabidopsis microRNAs: evidence for frequent birth and death of MIRNA genes. PloS One 2: e219.

58. Golaiacoever M, Subacchi A, Bagnarelli P, Lamontanara A, Cattivelli L, et al. (2010) A computational-based update on miRNAs and their targets in barley (Hordeum vulgare L.). BMC Genomics 11: 595.

59. Lu S, Nie X, Wang L, Du X, Biradar SS, et al. (2012) Identification and Characterization of MicroRNAs from Barley (Hordeum vulgare L.) by High-Throughput Sequencing. International Journal of Molecular Sciences 13: 2973–2984.

60. Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, et al. (2002) Prediction of perfect and near perfect microRNA hairpins. Genome research 12: 165–175.

61. Wu M-F, Tian Q, Reed JW (2006) Arabidopsis microRNA167 controls patterns of ARF6 and ARF8 expression, and regulates both female and male reproductive development. Development 133: 4211–4218.

62. Zhang J, Wang L, Mao Y, Cai W, Xue H, et al. (2005) Control of Root Cap Formation by MicroRNA156-Targeted Auxin-dependent transcription factors in Arabidopsis. The Plant Cell 17: 2204–2216.

63. Kleinow T, Bhalaria R, Bruer F, Umeda M, Salchert K, et al. (2000) Functional identification of an Arabidopsis miR ortholog by screening for heterologous multicopy suppression or deficiency in yeast. 25: 111–122.

64. Xu C, Jing R, Mao X, Jia X, Chang C (2007) A wheat (Triticum aestivum) protein phosphatase 2A catalytic subunit gene provides enhanced drought tolerance in tobacco. Annals of botany 99: 439–450.