Analysis of miRNAs and Their Targets during Adventitious Shoot Organogenesis of *Acacia crassicarpa*

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**Abstract**

Organogenesis is an important process for plant regeneration by tissue or cell mass differentiation to regenerate a complete plant. MicroRNAs (miRNAs) play an essential role in regulating plant development by mediating target genes at transcriptional and post-transcriptional levels, but the diversity of miRNAs and their potential roles in organogenesis of *Acacia crassicarpa* have rarely been investigated. In this study, approximately 10 million sequence reads were obtained from a small RNA library, from which 189 conserved miRNAs from 57 miRNA families, and 7 novel miRNAs from 5 families, were identified from *A. crassicarpa* organogenetic tissues. Target prediction for these miRNAs yielded 237 potentially unique genes, of which 207 received target Gene Ontology annotations. On the basis of a bioinformatic analysis, one novel and 13 conserved miRNAs were selected to investigate their possible roles in *A. crassicarpa* organogenesis by qRT-PCR. The stage-specific expression patterns of the miRNAs provided information on their possible regulatory functions, including shoot bud formation, modulated function after transfer of the culture to light, and regulatory roles during induction of organogenesis. This study is the first to investigate miRNAs associated with *A. crassicarpa* organogenesis. The results provide a foundation for further characterization of miRNA expression profiles and roles in the regulation of diverse physiological pathways during adventitious shoot organogenesis of *A. crassicarpa*.

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**Introduction**

With the characteristics of rapid growth, high pulp yield, high fiber quality and ability to thrive in degraded soils [1], *Acacia crassicarpa* has become an important economic species of the Fabaceae family, and has been planted for reforestation, reclamation of wasteland, and industrial material production on a large scale in Southeast Asia [2–3]. However, its recalcitrance of regeneration may be quite poor in natural habitats [9]. Therefore, a thorough understanding of the molecular mechanisms and gene regulatory networks involved in organogenesis of leguminous trees is essential in order to achieve improved *in vitro* plant regeneration and genetic transformation frequencies. Although important results have been obtained regarding the hormonal regulation of organogenesis and organ-related expression of genes and proteins in leguminous trees, few studies have focused on identification and expression of micro-RNAs (miRNAs) during organogenesis.

MiRNAs are small endogenous non-coding RNA usually associated with gene silencing by guiding cleavage of complementary miRNAs or suppression of translation [10]. The importance of miRNAs has been realized owing to their wide occurrence in all kinds of organisms and their important biological function in regulation of gene expression [11–12]. Increasing evidence shows that the length of 20–24 nt miRNAs plays a crucial role in developmental and physiological processes, including developmental timing, tissue-specific development, and stem cell maintenance and differentiation [13–14]. Plant organogenesis and somatic embryogenesis are the most important strategies for *in vitro* plant regeneration [15]. During the last decade, several reports have demonstrated the crucial roles played by miRNAs during somatic embryogenesis. Thus, miRNA-mediated modulation of somatic embryogenesis is closely associated with the plant regeneration process [16–17]. Plant organogenesis, which is also
Figure 1. Morphology of different stages during plant organogenesis in Acacia crassicarpa. S1: Zygotic embryo, excised from the mature seeds. S2: Zygotic embryo differentiated, after two weeks subculture. S3: Embryogenic callus, after three weeks subculture. S4: Shoot buds, after four weeks subculture. S5: Clusters of adventitious shoots, after 5 weeks subculture. S6: Adventitious shoot elongation. S1–S4 were cultured on MS medium containing 4.54 μM TDZ and 2.85 μM IAA, observed using a Leica stereomicroscope. S5–S6 were cultured on MS medium supplemented with 2.89 μM GA3.

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Materials and Methods

Plant Materials

The experimental materials were obtained at different developmental stages of an adventitious bud regeneration system induced from mature zygotic embryos of A. crassicarpa, as described by Yao et al. [18] (Figure 1). Based on morphological and anatomical traits, six developmental stages during shoot regeneration were defined and sampled. Generation of embryogenic callus (Figure 1, S1–S3) was induced in the dark and differentiation of shoot buds (Figure 1, S4–S6) occurred under light. All samples were frozen immediately in liquid nitrogen and stored at −80°C.

Extraction of Total RNA, Construction of sRNA Library and Solexa Sequencing

Total RNA at the six developmental stages was extracted using the mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA) in accordance with the manufacturer's instructions. The quality of the isolated total RNA was assessed by analysis with a NanoDrop ND-2000 spectrophotometer (NanoDrop, Wilmington, DE, USA). The integrity of the total RNA was monitored with a BioAnalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, Palo Alto, CA, USA). The RNA integrity number was higher than 6.0, therefore the isolated total RNA was suitable for library preparation.

Equal amounts of total RNA at each developmental stage were pooled. About 30 μg of total RNA was used for library construction and sequencing. A small RNA library was generated using the Illumina TruSeq Small RNA Sample Prep Kit (Illumina, Hayward, CA, USA) in accordance with the manufacturer's instructions. The purified small RNA fractions between 10 and 40 nt were ligated with proprietary 5’ and 3’ adaptors separately, and then converted to cDNA by RT-PCR. The purified cDNA library was used for cluster generation on an Illumina Cluster Station and then sequenced with an Illumina GAIIx platform following the manufacturer's instructions.

Bioinformatic Analysis of Sequencing Data

Raw sequencing reads were obtained using Sequencing Control Studio version 2.8 software (Illumina) following real-time sequencing image analysis. The extracted sequencing reads were first processed with a proprietary pipeline script, ACGT101-miR v4.2 (LC Sciences, Houston, TX, USA), for removal of 'impurity' and unmappable sequences resulting from sample preparation, sequencing chemistry and processes, and the optical digital resolution of the sequencer detector. The processed raw data have been uploaded to Short Read Archive of NCBI with the accession number of SRX469994. The remaining sequences with lengths of 15 to 30 nt were mapped to precursor miRNAs (pre-miRNAs) sequences of all plants in miRBase 19.0, for all mature miRNAs during Organogenesis of Acacia crassicarpa

| Number | miRNA name | Primer sequence (5’-3’) |
|--------|------------|------------------------|
| 1      | acr-miR159a-3p | GCTTGGATTGAAGGAGGCTCTAA |
| 2      | acr-miR162    | GCTCGATAAACCTCTGACATCCA |
| 3      | acr-miR390a-5p | CTCAGGAGGGATAGGCGCA |
| 4      | acr-miR396    | CCCCCACAGCTTGTAGACCTGA |
| 5      | acr-miR319a   | GCTTGGACTGAAGGGAGCTC |
| 6      | acr-miR156r   | GCCGCGTTGACAGAAGATAGAGCGATATAA |
| 7      | acr-miR164a   | GGGAAGCAGGGGACGCTGCA |
| 8      | acr-miR166h-3p | GCTCTCGGACACGGCTCTTCC |
| 9      | acr-miR167a   | GCCGTAAACTCCGACAGCATCTAAGGAA |
| 10     | acr-miR168a   | CGCTTCGTTGAAGGTCGGGAA |
| 11     | acr-miR171p-3p | GGGTATTGGACGCGCTGCAATATCA |
| 12     | acr-miR397a-5p | GGGTACCTGAGTGGCAGGCTGATGAA |
| 13     | acr-miR398d   | GCCGTGCTCTAGGGTGAGC |
| 14     | acr-novel2    | GCTCTCGGAAGCTCCCATACC |
| 15     | 5.8S rRNA    | CGCCCTGGCTGGTGACAA |

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miRNAs included in the database. The mapping sequences were further aligned against *Acacia mangium* expressed sequence tags (ESTs) and the genomic sequence of selected plant species, including *Arabidopsis thaliana*, *Populus trichocarpa* and soybean, to identify conserved miRNAs in *A. crassicarpa*. The unmapped sequences matching sequences in other defined databases, mainly comprising miRNA, RFam, and Repbase, were removed. A BLAST search with the remaining sequences against the selected genome such as *Arabidopsis thaliana*, *Populus trichocarpa* and soybean and ESTs of *Acacia mangium* was performed, and those extended sequences at the mapped genome positions with the propensity to form hairpins (as predicted with Mfold software) were selected as putative novel miRNAs.

**Prediction of Potential Target mRNAs**

Target prediction for the miRNAs was based on the principle of nearly perfect complementation between the miRNA and target mRNAs [19]. The identified conserved and putative novel miRNAs were all submitted for target gene prediction using TargetFinder (http://hercules.tigem.it/TargetFinder.html) with the default parameters, using the algorithm described by Quandt et al [20]. The target genes were then validated using public resources of The Arabidopsis Information Resource (TAIR; http://arabidopsis.org/). Sequences with a score of less than 4 were regarded as miRNA target genes. Given that many targets have more than one GO hit, the number of total hits can be higher than the total number of targets.

**Quantitative Real-time PCR**

Small RNAs of *A. crassicarpa* at the six organogenesis stages were isolated using micro RNA Extraction Kit (BioTeKe, Beijing, China) following the manufacturer's instructions. With minor modifications, poly (A)-tailed quantitative real-time PCR (qRT-PCR) was chosen for experimental identification. miRNA qRT-PCR was carried out using the NCodeTM VILO™ miRNA cDNA Synthesis Kit (Invitrogen, Carlsbad, USA) and SYBR Premix Ex TaqTM (Invitrogen, Carlsbad, USA) with the small RNAs as the template. Investigated miRNA was amplified using forward primers that were designed based on mature miRNA sequences (Table 1) and reverse primers provided with the kit. The qRT-PCR amplification protocol was as follows: initial activation at 94°C for 2 min, followed by 40 cycles consisting of 94°C for 20 s and 60°C for 34 s. To verify the absence of contamination, negative controls (no cDNA template) were carried out. The experiments were performed for five technique replicates and three biological replicates. The data were calculated using the 2^–ΔΔCt method [21]. 5.8S rRNA cloned from *A. crassicarpa* was selected as the reference gene for normalization.

**Statistical Analysis**

The statistical analysis was performed using one-way Between-groups Linkage with SPSS 18.0 (SPSS Taiwan Corp., Taiwan).

**Results**

**Small RNA Sequence Profile**

A small RNA library was constructed using pooled RNAs isolated at six developmental stages of adventitious shoot regeneration, and sequenced using Solexa high-throughput technology in a single lane. More than 10 million raw reads were yielded, which were filtered for adaptors and junk contaminants. After removal of redundant sequences we obtained 900,504 unique sequences ranging from 15 to 30 nt in length, of which the majority was 19–26 nt long. A major peak in the abundance of mapped small RNAs was observed at 24 nt, which accounted for 55.3% of the sequences (Figure 2). Those sequences were perfectly mapped to pre-miRNAs of miRBase 19.0 and selected species genomes including *Arabidopsis thaliana*, *Populus trichocarpa* and soybean or *Acacia mangium* ESTs. The generated sequence was deemed a ‘mappable sequence’. The unmappable reads were further searched against noncoding RNAs (rRNA, tRNA, snRNA, and snoRNA) deposited in the RFam and Repbase databases and comprised only a small fraction (4.59%) of the counts of all sequences, and were removed to avoid influencing miRNA identification (Table 2). Finally, 2,602,396 cleaned sequences representing 729,967 unique reads were used for subsequent analysis.

**Conserved miRNA Families and Isoforms**

To identify conserved miRNAs in *A. crassicarpa*, we selected the 18–25 nt mappable small RNAs for further analysis by observation of miRNA length in miRBase. From a series of BLAST
searches against miRBase 19.0 and Arabidopsis thaliana, Populus trichocarpa and soybean genomes or Acacia mangium or ESTs, we obtained 189 conserved unique sequences from 57 miRNA families allowing for sequence mismatches of not more than two nucleotides (Table 3). Among these sequences, the bulk of the miRNA families were conserved as we found orthologues of known miRNAs from all other plant species, such as miRNA families were conserved as we found orthologues of nucleotides (Table 3). Among these sequences, the bulk of the families allowing for sequence mismatches of not more than two

obtained 189 conserved unique sequences from 57 miRNA and soybean genomes or

trichocarpa

Figure 3. First nucleotide bias of identified miRNAs in

Arabidopsis thaliana

searches against miRBase 19.0 and

Acacia crassicarpa.

(A) Conserved miRNAs, (B) novel miRNA candidates.

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Putative Novel miRNAs

The sequences unmapped to the pre-miRNAs in miRBase 19.0 were chosen to identify putative novel miRNAs. The following criteria were applied: (1) the unique sequences were mapped to the selected species genomes; (2) the extended sequence at the mapped genome location had the propensity to form a hairpin. Taken together, a total of 7 novel miRNA candidates from 5 families were obtained with a minimal folding free energy index (MFEI) for the hairpin structure of the miRNA precursor of less than 0.7 (Table 4). These miRNA candidates commonly displayed a strong bias toward a 5’ U at the first nucleotide position (Figure 3) and stem-loop structure precursors (Figure 4), which was consistent with conserved miRNAs [23]. Some previous studies reported that a standard for discrimination between high-confidence microRNAs and fragments of other RNAs in deep sequencing data was the detection of miRNA* sequences [25]. Among the miRNA candidates in the present study, four novel miRNAs were identified with their complementary miRNA* belonging to two miRNA families, which implied that these putative miRNAs were most likely novel to A. crassicarpa, and several potentially novel miRNAs might be specific to A. crassicarpa or Fabaceae species. Of these potentially novel miRNAs, the copy number of several miRNA* was low. This feature is consistent with miRNA* degradation during the miRNA generation procedure [24], so counts of miRNA* were not included in the most recent criteria for annotation of plant miRNAs. In the present study, acr-novel2* showed higher counts than the corresponding novel miRNAs. This finding was in accordance with the recent discovery by Zhang et al. [25] that miRNA* sequences are abundantly expressed.

Table 2. Summary of reads from raw data to cleaned sequences of Acacia crassicarpa.

| Category      | Total reads | Unique reads |
|---------------|-------------|--------------|
| raw reads     | 10,378,615  | 100%         | 980,584      | 100%        |
| filtered reads| 7,315,987   | 70.49%       | 203,692      | 20.78%      |
| Repeats       | 449,801     | 4.33%        | 44,866       | 4.58%       |
| clean reads   | 2,602,396   | 25.07%       | 729,967      | 74.44%      |

Note: filtered reads: 5’ adapter, 3’ adapter, 5’ adapter and 3’ adapter joined together without insertion length with <15 nt and >32 nt and junk reads.

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The abundance of known miRNA families was also monitored. Interestingly, the number of isoforms in that differed in no more than two nucleotides, which are regarded as miRNA isoforms [22]. Interestingly, the number of isoforms in differently conserved miRNA families varied considerably. Four families (miR156, miR166, miR167, and miR396) contained the highest number of multiple isoforms with 13, 11, 10, and 10 members, respectively. Eighteen miRNA families (e.g., miR156, miR170, miR393, miR399, and miR1511) contained only one member.

The abundance of known miRNA families was also monitored. Interestingly, a notable divergence in expression frequency among miRNAs during shoot regeneration was observed. For example, miR159, miR166, and miR396 were detected most frequently with the counts of 15,425, 11,560, and 15,290, respectively; miR156, miR157, and miR319 showed moderate abundance with counts of 4355, 3566, and 4020, respectively; and miRNAs such as miR2592, miR5256, miR5740, and miR6485 showed low abundance (Table 3).

Figure 3. First nucleotide bias of identified miRNAs in Acacia crassicarpa. (A) Conserved miRNAs, (B) novel miRNA candidates.

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| miRNA family | Gene name | Length | Sequence | Counts | Reference miRNA |
|--------------|-----------|--------|----------|--------|-----------------|
| MIR156       | acr-miR156a | 20     | UGACAGAAGAGAGAGCAC | 1       | mtr-miR156a |
|              | acr-miR156b | 20     | UGACAGAAGAGAGUGACAU | 9       | cca-miR156b |
|              | acr-miR156c | 23     | UGACAGAAGAGAGAGCACCCA | 1       | ghr-miR156c |
|              | acr-miR156d | 20     | UGACAGAAGAGAGUGACAC | 584     | gma-miR156a |
|              | acr-miR156e | 21     | UUGACAGAAGAGAGUCACGAC | 80      | sbi-miR156e |
|              | acr-miR156e-p3 | 21     | GCUCUCUUUUCUCUGUCAUC | 44      | tcc-MIR156e-p3 |
|              | acr-miR156f | 21     | UUGACAGAAGAGAGUGACAC | 17      | gma-miR156b |
|              | acr-miR156g | 21     | UUGACAGAAGAGAGAGACAA | 5       | ath-miR156g |
|              | acr-miR156h-5p | 21    | UUGACAGAAGAGAGAGACAA | 5       | mtr-MIR156i-p3 |
|              | acr-miR156i-3p | 22    | GCUCUCUUUUCUCUGUCAUC | 15030   | gma-MIR156i-p3 |
|              | acr-miR156j | 21     | UUGACAGAAGAGAGAGACAA | 4       | gma-miR156j |
|              | acr-miR156k | 21     | UUGACAGAAGAGAGAGACCA | 58      | gma-miR156k |
|              | acr-miR156l | 21     | UUGACAGAAGAGAGAGACCA | 9       | ath-MIR156l-p3 |
|              | acr-miR156m | 21     | UUGACAGAAGAGAGAGACCA | 3530    | gma-miR156p |
|              | acr-miR156n | 21     | UUGACAGAAGAGAGAGACCA | 7       | mtr-miR156g |
|              | acr-miR156o-3p | 22   | GCUCUCUUUUCUCUGUCAUC | 18      | ptc-MIR156b-p5 |
|              | acr-miR156p | 21     | UUGACAGAAGAGAGAGACCA | 127     | ath-miR156c |
|              | acr-miR156q | 19     | UUGACAGAAGAGAGAGACCA | 8       | ptc-MIR156d |
|              | acr-miR156r | 21     | UUGACAGAAGAGAGAGACCC | 195     | ath-miR156b |
|              | acr-miR156s | 21     | UUGACAGAAGAGAGAGACCC | 133     | gma-miR156a |
|              | acr-miR156t | 21     | UUGACAGAAGAGAGAGACCC | 7       | gma-miR156a |
|              | acr-miR156u | 21     | UUGACAGAAGAGAGAGACCC | 1       | ath-MIR160a-p3 |
|              | acr-miR156v | 21     | UUGACAGAAGAGAGAGACCC | 234     | aau-miR162 |
|              | acr-miR156w | 21     | UUGACAGAAGAGAGAGACCC | 3       | aau-MIR162-p5 |
|              | acr-miR156x | 21     | UUGACAGAAGAGAGAGACCC | 49      | gma-miR164a |
|              | acr-miR156y | 21     | UUGACAGAAGAGAGAGACCC | 4       | aly-miR164c-5p |
|              | acr-miR156z | 21     | UUGACAGAAGAGAGAGACCC | 7       | aly-miR165a-3p |
|              | acr-miR156aa | 23    | GCAAUGUUGAGCGGAGGAUCAGAU | 3       | gma-miR156a-5p |
|              | acr-miR156ab | 21    | GCGUAUGAGGAGCCAAGAUAA | 18      | ptc-MIR166b-p5 |
|              | acr-miR156ac | 19    | UUGACAGAAGAGAGAGACCC | 11      | gma-MIR166i-5p |
|              | acr-miR156ad | 21    | UUGACAGAAGAGAGAGACCC | 16      | gma-MIR166e-p5 |
|              | acr-miR156ae | 21    | UUGACAGAAGAGAGAGACCC | 2271    | gma-miR166h-3p |
|              | acr-miR156af | 21    | UUGACAGAAGAGAGAGACCC | 11      | gma-MIR166i-5p |
|              | acr-miR156ag | 21    | UUGACAGAAGAGAGAGACCC | 1       | ptc-MIR166g-5p |

Table 3. Identification of conserved miRNA families in *Acacia crassicarpa* with corresponding isoforms.
Table 3. Cont.

| miRNA family | Gene name | Length | Sequence | Counts | Reference miRNA |
|--------------|-----------|--------|----------|--------|-----------------|
| MIR167       | acr-miR167a | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 64     | gma-miR167a     |
|              | acr-miR167a-3p | 23   | GGCAUGGCUAGCAGCUACUCU   | 5      | gma-MIR167a-p3  |
|              | acr-miR167b-5p | 23   | UGAAGCUGCCAGCAUAGAUCAU   | 6      | csi-MIR167b-p3  |
|              | acr-miR167b-5p | 23   | UGAAGCUGCCAGCAUAGAUCAU   | 3      | mtr-miR167b-5p  |
|              | acr-miR167c | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 3      | vvi-miR167c     |
|              | acr-miR167d | 22     | UGAAGCUGCCAGCAUAGAUCAU   | 266    | gma-miR167d     |
|              | acr-miR167e | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 9      | gma-miR167e     |
|              | acr-miR167e-3p | 20   | UGAAGCUGCCAGCAUAGAUCAU   | 4      | dpr-miR167e     |
|              | acr-miR167f | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 5      | mtr-miR167f     |
|              | acr-miR167g | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 137    | gma-miR167g     |
| MIR168       | acr-miR168a | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 456    | gma-miR168a     |
|              | acr-miR168a-3p | 21   | UGAAGCUGCCAGCAUAGAUCAU   | 322    | gma-MIR168a-p3  |
|              | acr-miR168b | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 2      | mtr-miR168b     |
|              | acr-miR168b-3p | 22   | UGAAGCUGCCAGCAUAGAUCAU   | 8      | nta-MIR168a-p3  |
|              | acr-miR169a | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 5      | gma-miR169a     |
|              | acr-miR169a-3p | 23   | UGAAGCUGCCAGCAUAGAUCAU   | 1      | ath-MIR169a     |
|              | acr-miR169a-3p | 23   | UGAAGCUGCCAGCAUAGAUCAU   | 1      | ath-MIR169a     |
|              | acr-miR169b | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 5      | gma-miR169b     |
|              | acr-miR169c | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 2      | mtr-miR169c     |
|              | acr-miR169e | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 5      | gma-miR169e     |
|              | acr-miR169f | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 4      | gma-miR169f     |
|              | acr-miR169g | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 49     | gma-miR169g     |
|              | acr-miR169h | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 16     | vvi-miR169h     |
|              | acr-miR169i | 19     | UGAAGCUGCCAGCAUAGAUCAU   | 1      | gma-miR169i     |
|              | acr-miR169j | 22     | UGAAGCUGCCAGCAUAGAUCAU   | 12     | gma-miR169j     |
|              | acr-miR169k | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 8      | gma-miR169k     |
|              | acr-miR169l | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 9      | gma-miR169l     |
|              | acr-miR169m | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 3      | mtr-miR169m     |
|              | acr-miR169n | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 1      | cme-miR169n     |
|              | acr-miR171a | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 7      | gma-miR171a     |
|              | acr-miR171b | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 9      | gma-miR171b     |
|              | acr-miR171c | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 3      | mtr-miR171c     |
|              | acr-miR171d | 22     | UGAAGCUGCCAGCAUAGAUCAU   | 1      | zma-miR171d     |
|              | acr-miR171f | 22     | UGAAGCUGCCAGCAUAGAUCAU   | 5      | gma-miR171f     |
|              | acr-miR171g | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 12     | gma-miR171g     |
|              | acr-miR171h | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 8      | gma-miR171h     |
|              | acr-miR171i | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 1      | gma-miR171i     |
|              | acr-miR171j | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 11     | gma-miR171j     |
|              | acr-miR172a | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 3      | gma-miR172a     |
|              | acr-miR172b | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 49     | gma-miR172b     |
|              | acr-miR172c | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 7      | ptc-MIR172c-p5  |
|              | acr-miR172d | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 45     | gma-miR172d     |
|              | acr-miR172e | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 7      | cme-miR172e     |
|              | acr-miR172f | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 8      | mtr-miR172f     |
|              | acr-miR172g | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 26     | gma-miR172g     |
|              | acr-miR172h | 21     | UGAAGCUGCCAGCAUAGAUCAU   | 912    | gma-miR172h     |

miRNAs during Organogenesis of *Acacia crassicarpa*
Table 3. Cont.

| miRNA family | Gene name | Length | Sequence | Counts | Reference miRNA |
|--------------|-----------|--------|----------|--------|-----------------|
| MIR2590      | acr-miR2590d-5p | 18     | UCAGCUGUGCUAGAAAUC | 1      | mtr-MIR2590d-p5 |
| MIR2592      | acr-miR2592bj-5p | 18     | AUUCCACUGUCCUGUCCUG | 3      | mtr-MIR2592bj-p5 |
| MIR2631      | acr-miR2631-3p | 19     | UUAUUAUUGAAAAUUGUG | 2      | mtr-MIR2631-p3  |
| MIR2655      | acr-miR2655n-5p | 21     | UUUAUUAUUGAAAAUUGUG | 2      | mtr-MIR2655n-p5 |
|              | acr-miR2655o-3p | 21     | UUUAUUAUUGAAAAUUGUG | 2      | mtr-MIR2655o-p3 |
| MIR2911      | acr-miR2911    | 23     | GGGCGCGGCGGCGGCGGCGG | 314    | han-miR2911     |
|              | acr-miR2911-5p | 23     | GGGCGCGGCGGCGGCGGCGG | 500    | han-MIR2911-p5  |
| MIR2916      | acr-miR2916-5p | 24     | CUAGCUUUAACCAUAAUUGUC | 42     | pti-MIR2916-p5  |
|              | acr-miR2916-3p | 18     | GCCUUGUCUUCUCAACCAUAAUUGUC | 31     | pti-MIR2916-p3  |
| MIR319       | acr-miR319    | 25     | UUUGACUGAAGAGGAGGCUGUUCUAAUUU | 2      | tcc-miR319     |
|              | acr-miR319a   | 20     | UUUGACUGAAGAGGAGGCUGUUCUAAUUU | 3259   | gma-miR319a    |
|              | acr-miR319f   | 19     | UUUGACUGAAGAGGAGGCUGUUCUAAUUU | 43     | gma-miR319g    |
|              | acr-miR319g   | 21     | UUUGACUGAAGAGGAGGCUGUUCUAAUUU | 689    | gma-miR319g    |
|              | acr-miR319h-5p | 21    | AGCUUGCUACUCAUAAUUGUC | 12     | gma-MIR319h-p5 |
|              | acr-miR319i   | 21     | UUUGACUGAAGAGGAGGCUGUUCUAAUUU | 17     | pti-miR319i    |
|              | acr-miR319n   | 21     | UUUGACCGAAGAGGAGGCUGUUCUAAUUU | 1      | gma-miR319n    |
| MIR390       | acr-miR390a   | 22     | AAGCUUGCUAAGAGGAGGCUGUUCUAAUUU | 3      | cme-miR390a    |
|              | acr-miR390a-3p | 21    | AAGCUUGCUAAGAGGAGGCUGUUCUAAUUU | 1      | gma-miR390a-3p |
|              | acr-miR390a-5p | 21    | AAGCUUGCUAAGAGGAGGCUGUUCUAAUUU | 398    | gma-miR390a-5p |
|              | acr-miR390g-3p | 21    | AAGCUUGCUAAGAGGAGGCUGUUCUAAUUU | 3      | gma-MIR390g-p3 |
| MIR393       | acr-miR393a   | 22     | UCAAGGAGGCUAGCAGCAUGAUUC | 9      | gma-miR393a    |
|              | acr-miR393b-3p | 21    | UAGGACUGAAGGGGAUGCUUCUAAUUU | 1      | gma-MIR393b-p3 |
|              | acr-miR393i-3p | 21    | UAGGACUGAAGGGGAUGCUUCUAAUUU | 2      | gma-MIR393i-p3 |
| MIR394       | acr-miR394a-5p | 20    | UGGCAUGAAGGGAUGCUUCUAAUUU | 69     | gma-miR394a-5p |
| MIR395       | acr-miR395a   | 21     | UGAGAUGCUUUUGGGGAAGCUUCUAAUUU | 104    | gma-miR395a    |
|              | acr-miR395a-5p | 18    | UGAGAUGCUUUUGGGGAAGCUUCUAAUUU | 2      | gma-MIR395a-p5 |
|              | acr-miR395b   | 21     | CUGAAGGCUUUGGGGAAGCUUCUAAUUU | 2      | cca-miR395b    |
|              | acr-miR395c   | 21     | CUGAAGGCUUUGGGGAAGCUUCUAAUUU | 24     | pti-miR395b    |
|              | acr-miR395d   | 21     | CUGAAGGCUUUGGGGAAGCUUCUAAUUU | 1      | gma-miR395d    |
|              | acr-miR395g-5p | 21    | AGUUCGCAGAAACGCUUCUAAUUU | 63     | gma-MIR395g-p5 |
|              | acr-miR395h-5p | 21    | AGUUCGCAGAAACGCUUCUAAUUU | 1      | gma-MIR395h-p5 |
|              | acr-miR395i   | 20     | UGAGAUGCUUUUGGGGAAGCUUCUAAUUU | 8      | gma-MIR395i    |
| MIR396       | acr-miR396    | 22     | UUUCACAGCUUUUCUCAAGCUUGC | 7      | dpr-miR396     |
|              | acr-miR396c   | 21     | UUUCACAGCUUUUCUCAAGCUUGC | 1334   | aau-miR396     |
|              | acr-miR396-3p | 21     | UUUCACAGCUUUUCUCAAGCUUGC | 167    | aau-MIR396-p3  |
|              | acr-miR396a-5p | 22    | UUUCACAGCUUUUCUCAAGCUUGC | 56     | gma-miR396a-5p |
|              | acr-miR396a-3p | 21    | UUUCACAGCUUUUCUCAAGCUUGC | 13     | csi-MIR396a-p3 |
|              | acr-miR396b-5p | 21    | UUUCACAGCUUUUCUCAAGCUUGC | 261    | gma-miR396b-5p |
|              | acr-miR396b-3p | 21    | UUUCACAGCUUUUCUCAAGCUUGC | 29     | gma-miR396b-3p |
|              | acr-miR396e   | 22     | UUUCACAGCUUUUCUCAAGCUUGC | 32     | cme-miR396e    |
|              | acr-miR396f   | 21     | UUUCACAGCUUUUCUCAAGCUUGC | 1388   | pti-miR396f    |
|              | acr-miR396g-5p | 19    | UUUCACAGCUUUUCUCAAGCUUGC | 2      | pti-miR396g-5p |
|              | acr-miR396j-5p | 21    | UUUCACAGCUUUUCUCAAGCUUGC | 3      | gma-MIR396j-p5 |
| MIR397       | acr-miR397a   | 21     | AUUGAUGCUAGCGCUUGAAGA | 28     | gma-miR397a    |
|              | acr-miR397a-5p | 21    | AUUGAUGCUAGCGCUUGAAGA | 12     | aly-miR397a-5p |
|              | acr-miR397b-3p | 21    | AUUGAUGCUAGCGCUUGAAGA | 43     | gma-miR397b-3p |
|              | acr-miR397c   | 21     | AUUGAUGCUAGCGCUUGAAGA | 1      | pti-miR397c    |
| MIR398       | acr-miR398    | 22     | UGGUUCUGAAGCGCGCGGGCGGCGGCGG | 3      | nta-miR398     |
|              | acr-miR398a   | 23     | UGGUUCUGAAGCGCGCGGGCGGCGGCGG | 1      | bdi-miR398a    |
Putative Target Prediction for Conserved and Novel miRNAs

Putative targets of identified conserved and novel miRNAs were predicted by BLAST using mRNA sequences from *A. thaliana* [22] using TargetFinder. On the basis of completely complementarity between the miRNA and target mRNA [26], we screened 237 genes for feasible targets with a total score not higher than 3 (Table 5). Among the targets predicted, the most frequent were transcription factors, including a SBP transcription factor, MYB transcription factor, NAC transcription factor and GRF transcription factor, and other putative target genes involved in a range of physiological or metabolic processes, for example functional proteins such as protein kinases, laccase, and protein phosphatase. Many reports have demonstrated that target genes of conserved miRNAs are consistent among the majority of plant species [27], and this was the case in the present study. Putative target genes of Table 3.

| miRNA family | Gene name | Length | Sequence | Counts | Reference |
|--------------|-----------|--------|----------|--------|-----------|
| acr-miR398d |           | 21     | UGUGUUCAGGAGGGCCCUU       | 4      | gma-miR398a |
| acr-miR398b |           | 21     | UGUGUUCAGGAGGCCCCUUG      | 1      | ath-miR398b |
| acr-miR398c |           | 21     | UGUGUUCAGGAGGCCCCCUU      | 181    | gma-miR398c |
| MIR399      | acr-miR399a | 20     | UGUGUUCAGGAGGCCCCUUG      | 1      | gma-miR399a |
|             | acr-miR399b | 21     | UGUGUUCAGGAGGCCCCUUG      | 2      | vun-miR399b |
|             | acr-miR399d | 21     | UGUGUUCAGGAGGCCCCUUG      | 8      | cme-miR399d |
| MIR403      | acr-miR403-3p | 21   | UUUAUUUCAGGAGGCCCCUUG     | 975    | aly-miR403-3p |
|             | acr-miR403a | 19     | UUUAUUUCAGGAGGCCCCUUG     | 3      | gma-miR403a |
| MIR408      | acr-miR408-5p | 21   | CUGGAGAGGCCCCUUG          | 44     | ptc-miR408-5p |
|             | acr-miR408a-3p | 21   | CUGGAGAGGCCCCUUG          | 157    | gma-miR408a-3p |
|             | acr-miR408b-3p | 21  | CUGGAGAGGCCCCUUG          | 19     | gma-MIR408b-3p |
| MIR4414     | acr-miR4414b | 21    | UUUAUUUCAGGAGGCCCCUUG     | 5      | mtr-miR4414b |
| MIR4415     | acr-miR4415a-3p | 21 | UUUAUUUCAGGAGGCCCCUUG     | 305    | gma-MIR4415a-3p |
| MIR4416     | acr-miR4416c-3p | 21 | UUUAUUUCAGGAGGCCCCUUG     | 2      | gma-MIR4416c-3p |
| MIR482      | acr-miR482-5p | 21   | CUGGAGAGGCCCCUUG          | 1      | ptc-miR482-5p |
|             | acr-miR482a-3p | 21   | CUGGAGAGGCCCCUUG          | 2      | gma-MIR482a-3p |
| MIR4995     | acr-miR4995-5p | 20   | UUUAUUUCAGGAGGCCCCUUG     | 5      | gma-MIR4995-5p |
| MIR5015     | acr-miR5015-5p | 24   | UUUAUUUCAGGAGGCCCCUUG     | 1      | ath-MIR5015-5p |
| MIR5030     | acr-miR5030-3p | 20   | UUUAUUUCAGGAGGCCCCUUG     | 1      | gma-MIR5030-3p |
| MIR5139     | acr-miR5139 | 18     | AACCUGGCCCCUUGAACC       | 282    | rgl-miR5139 |
| MIR5248     | acr-miR5248 | 21     | UUUAUUUCAGGAGGCCCCUUG     | 2      | mtr-miR5248 |
| MIR5256     | acr-miR5256-5p | 21   | UUUAUUUCAGGAGGCCCCUUG     | 3      | mtr-MIR5256-5p |
| MIR5257     | acr-miR5257-5p | 21   | UUUAUUUCAGGAGGCCCCUUG     | 1      | mtr-MIR5257-5p |
| MIR5261     | acr-miR5261 | 21     | UUUAUUUCAGGAGGCCCCUUG     | 1      | mtr-MIR5261 |
| MIR5281     | acr-miR5281b | 24    | UUUAUUUCAGGAGGCCCCUUG     | 6      | mtr-MIR5281b |
|             | acr-miR5281c-5p | 23   | UUUAUUUCAGGAGGCCCCUUG     | 1      | mtr-MIR5281c-5p |
| MIR5282     | acr-miR5282-3p | 24   | GCAAACCGAGGCCCCUUGAAGU   | 1      | mtr-MIR5282-3p |
| MIR530      | acr-miR530a | 22     | UUUAUUUCAGGAGGCCCCUUG     | 1      | gma-MIR530a |
| MIR5368     | acr-miR5368-3p | 19   | GAACCACUCUGGAGGCCCCUUG    | 96     | gma-MIR5368-3p |
|             | acr-miR5368-5p | 20   | CCUGGAGGCCCCUUGAAGGCCCCUUG | 16 | gma-MIR5368-5p |
| MIR5740     | acr-miR5740-5p | 21   | UUUAUUUCAGGAGGCCCCUUG     | 3      | mtr-MIR5740-5p |
| MIR6478     | acr-miR6478 | 25     | CUGGCCCCUUGAAGGCCCCUUGAGG | 190   | ptc-miR6478 |
| MIR6485     | acr-miR6485-5p | 23   | UGUGGAGGCCCCUUGAAGGCCCCUUG | 3   | hbr-MIR6485-5p |
| MIR827      | acr-miR827 | 18     | UUUAUUUCAGGAGGCCCCUUG     | 1      | pUc-miR827 |
| MIR828      | acr-miR828 | 22     | UUUAUUUCAGGAGGCCCCUUG     | 1      | gma-miR828a |
| MIR858      | acr-miR858a | 21    | UUUAUUUCAGGAGGCCCCUUG     | 12     | ath-miR858a |
|             | acr-miR858b | 21    | UUUAUUUCAGGAGGCCCCUUG     | 7      | ath-miR858b |
| MIR869      | acr-miR869-5p | 18   | CCGGCCCCUUGAAGGCCCCUUGAAGG | 1    | ath-MIR869-5p |
| MIR1310     | acr-miR1310-3p | 20   | AAACUCUCUCUAAGGCCCCUUG    | 66     | han-MIR1310-3p |
| MIR1448     | acr-miR1448-3p | 20   | UUUAUUUCAGGAGGCCCCUUG     | 14     | ptc-MIR1448-3p |
| MIR1511     | acr-miR1511 | 18     | AAACUGGCCCCUUGAAGGCCCCUUGAAGG | 11   | gma-miR1511 |

Table 3. Cont.

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miR156/157 mainly encoded a SBP transcription factor family protein and SQUAMOSA promoter binding protein (SPL), which are involved in leaf and flower development. The miR160 target mRNA was annotated with an auxin response factor (ARF), which might be associated with auxin response during plant organogenesis [28]. These results provide valuable information for the further research.

To describe the gene functions, we classified the potential targets into three categories based on TAIR GO annotations: molecular functions, biological processes and cellular components. For the molecular function category, genes were assigned to eight subcategories (Figure 5A). The GO terms binding (35.06%), enzyme regulator activity (22.87%), and transcription factor activity (14.33%) were the most frequent, and especially RNA polymerase II transcription cofactor activity, which belonged to the transcription factor activity subcategory, is important for miRNA regulation [29]. Biological processes, which included 11 subcategories (Figure 5B), most frequently included GO terms involved in developmental processes (14.87%) and regulation of transcription (11.21%). Five subcategories of cellular components were identified (Figure 5C), of which the most frequent were nucleus (20.64%) and membrane (12.22%). Thus, the high frequency of GO terms associated with developmental processes indicated that many of the miRNAs identified in this study were involved in *A. crassicarpa* organogenesis by regulating molecular functions, biological processes and cellular components.

Expression Patterns of miRNAs During Organogenesis

To further investigate the role of miRNAs during *A. crassicarpa* organogenesis, 14 miRNAs of known function or high expression counts (one novel and 13 conserved [30–43]) (Table 6) were selected for analysis by qRT-PCR [44], which is a reliable method.

![Figure 4. Stem-loop structure of partial novel miRNA precursors.](https://doi.org/10.1371/journal.pone.0093438.g004)

**Table 4. Novel miRNA families identified from *Acacia crassicarpa*.**

| miRNA  | Location                     | Sequence (5’-3’)                | Length | Count | MFEI |
|--------|------------------------------|---------------------------------|--------|-------|------|
| acr-novel1 | gi|293578058:1:181 | AUUAUAGGAACACUUUUUGUAG | 21     | 22    | 1.6  |
| acr-novel1* |       | AAAAGUGUCCUAUAAUUGGACC | 24     | 4515  | 0.9  |
| acr-novel2 | gi|293579709:1:247 | UGUGGGACGCCUAGGAAGAG | 20     | 2     | 0.9  |
| acr-novel2* |       | UCUUUCUAAUUGCCUCUCCAUACC | 22     | 4515  | 0.9  |
| acr-novel3 | gi|293581175:360:620 | UCCGGUCUAAUAAUAGAAC | 21     | 7     | 1.6  |
| acr-novel4 | gma_Gm07:35780857:35781118 | AUCGACGCUUGACUCAUAU | 22     | 58    | 1    |
| acr-novel5 | ptr_Ch13:8496156:8496416 | UACUUAAGUAGGCAACC | 21     | 7     | 0.9  |

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Table 5. Predicted target genes of miRNAs in *Acacia crassicarpa*.

| miRNA   | Target      | Score | Annotation                                                                 |
|---------|-------------|-------|-----------------------------------------------------------------------------|
| MIR156  | AT1G69170   | 1     | SBP domain transcription factor family protein                              |
| AT5G50570 |            | 1     | SBP domain transcription factor family protein                              |
| AT5G50670 |            | 1     | SBP domain transcription factor family protein                              |
| AT1G35160 |            | 2     | squamosa promoter binding protein-like 4                                    |
| AT1G71690 |            | 3     | Protein of unknown function (DUF579)                                        |
| AT3G15270 |            | 3     | squamosa promoter binding protein-like 5                                    |
| AT5G10945 |            | 3     | MIR156D; miRNA                                                               |
| AT5G10946 |            | 3     | unknown protein                                                              |
| AT2G19420 |            | 0     | unknown protein                                                              |
| AT1G29900 |            | 2     | carbamoyl phosphate synthetase B                                             |
| AT2G33810 |            | 2     | squamosa promoter binding protein-like 3                                    |
| AT3G28690 |            | 2     | Protein kinase superfamily protein                                           |
| AT1G17500 |            | 3     | ATPase E1-E2 type family protein/haloacid dehalogenase-like hydrolase family protein |
| AT1G29030 |            | 3     | Apoptosis inhibitory protein 5 (API5)                                        |
| AT1G44790 |            | 3     | ChaC-like family protein                                                     |
| AT1G48410 |            | 3     | Stabilizer of iron transporter SufD/Poly nucleotidyl transferase             |
| AT1G61580 |            | 3     | R-protein L3 B                                                               |
| AT1G62305 |            | 3     | Core-2/A-branching beta-1,6-N-acetylglucosaminyltransferase family protein   |
| AT1G71240 |            | 3     | Plant protein of unknown function (DUF639)                                   |
| AT2G11910 |            | 3     | unknown protein                                                              |
| AT3G04870 |            | 3     | zeta-carotene desaturase                                                     |
| AT3G06670 |            | 3     | binding                                                                     |
| AT3G18210 |            | 3     | 2-oxoglutarate (2OG) and Fe(III)-dependent oxygenase superfamily protein     |
| AT3G25566 |            | 3     | transposable element gene                                                   |
| AT1G27360 |            | 0     | squamosa promoter-like 11                                                   |
| AT1G27370 |            | 0     | squamosa promoter binding protein-like 10                                   |
| AT2G42200 |            | 0     | squamosa promoter binding protein-like 9                                    |
| AT3G57920 |            | 0     | squamosa promoter binding protein-like 15                                   |
| AT5G43270 |            | 0     | squamosa promoter binding protein-like 2                                    |
| AT1G22000 |            | 3     | FBD, F-box and Leucine Rich Repeat domains containing protein               |
| AT3G44096 |            | 3     | transposable element gene                                                   |
| AT3G47170 |            | 3     | HXXXD-type acyl-transferase family protein                                  |
| AT3G66600 |            | 3     | Protein of unknown function, DUF547                                         |
| AT1G16916 |            | 3     | unknown protein                                                              |
| AT4G24270 |            | 3     | EMBRYO DEFECTIVE 140                                                        |
| AT5G11977 |            | 3     | MIR156E; miRNA                                                               |
| AT5G08620 |            | 3     | DEA(D/H)-box RNA helicase family protein                                     |
| AT5G24930 |            | 3     | CONSTANS-like 4                                                             |
| AT5G44280 |            | 3     | RING 1A                                                                     |
| AT5G55590 |            | 3     | Pectin lyase-like superfamily protein                                        |
| AT5G55835 |            | 3     | MIR156H; miRNA                                                               |
| AT3G20380 |            | 3     | phosphate transporter 4,5                                                    |
| AT1G48742 |            | 2     | MIR157D; miRNA                                                              |
| AT3G18217 |            | 2     | MIR157C; miRNA                                                              |
| miRNA   | Target                     | Score | Annotation                                                 |
|---------|----------------------------|-------|------------------------------------------------------------|
| AT1G30450 | cation-chloride co-transporter 1 |
| AT1G73650 | Protein of unknown function (DUF1295) |
| AT3G13490 | Lysyl-tRNA synthetase, class II |
| AT4G31050 | Biotin/lipoate A/B protein ligase family |
| AT5G35930 | AMP-dependent synthetase and ligase family protein |
| AT4G05400 | copper ion binding |
| MIR159  | unknown protein |
| AT4G34010 | 1-amino-cyclopropane-1-carboxylate synthase 8 |
| AT3G60460 | myb-like HTH transcriptional regulator family protein |
| AT4G22415 | transposable element gene |
| AT3G28915 | transposable element gene |
| AT3G3133 | transposable element gene |
| AT5G65540 | unknown protein |
| AT2G26950 | myb domain protein 104 |
| AT3G11440 | myb domain protein 65 |
| AT3G52030 | F-box family protein with WD40/YVTN repeat doamin |
| AT5G06100 | myb domain protein 33 |
| AT1G08030 | tyrosylprotein sulfotransferase |
| AT1G52100 | Mannose-binding lectin superfamily protein |
| AT1G76390 | ARM repeat superfamily protein |
| AT2G32460 | myb domain protein 101 |
| AT3G33076 | transposable element gene |
| AT3G33084 | transposable element gene |
| AT4G26930 | myb domain protein 97 |
| AT4G27330 | sporocyteless (SPL) |
| AT5G16810 | Protein kinase superfamily protein |
| AT5G28335 | transposable element gene |
| AT5G55020 | myb domain protein 120 |
| MIR160  | auxin response factor 10 |
| AT2G28350 | NAC domain containing protein 1 |
| AT1G56010 | NAC domain transcriptional regulator superfamily protein |
| AT3G15170 | NAC domain transcriptional regulator superfamily protein |
| AT5G53950 | NAC domain transcriptional regulator superfamily protein |
| MIR167  | MIR167C, miRNA |
| AT3G04765 | RING/U-box superfamily protein |
| AT3G07120 | nuclear factor Y, subunit A2 |
| AT5G06510 | nuclear factor Y, subunit A10 |
| AT1G68560 | alpha-xilosidase 1 |
| AT2G46020 | transcription regulatory protein SNF2, putative |
| AT5G23110 | Zinc finger, C3HC4 type (RING finger) family protein |
| AT1G17590 | nuclear factor Y, subunit A8 |
| AT1G54160 | nuclear factor Y, subunit A5 |
| AT5G42120 | Concanavalin A-like lectin protein kinase family protein |
| AT5G12840 | nuclear factor Y, subunit A1 |
| AT3G20910 | nuclear factor Y, subunit A9 |
| miRNA  | Target      | Score | Annotation                                      |
|--------|-------------|-------|------------------------------------------------|
| MIR171 | AT2G45160   | 1     | GRAS family transcription factor               |
|        | AT3G60630   | 1     | GRAS family transcription factor               |
|        | AT4G00150   | 1     | GRAS family transcription factor               |
|        | AT1G62035   | 3     | MIR171C; miRNA                                  |
| MIR172 | AT3G60120   | 1     | target of early activation tagged (EAT) 2      |
|        | AT5G65790   | 2     | myb domain protein 68                          |
|        | AT5G67180   | 2     | target of early activation tagged (EAT) 3      |
|        | AT1G61290   | 3     | syntaxin of plants 124                         |
|        | AT3G11435   | 3     | MIR172C; miRNA                                  |
|        | AT5G12900   | 3     | unknown protein                                 |
|        | AT4G36920   | 1     | Integrase-type DNA-binding superfamily protein  |
|        | AT5G60120   | 2     | target of early activation tagged (EAT) 2      |
|        | AT5G65790   | 2     | myb domain protein 68                          |
|        | AT5G67180   | 2     | target of early activation tagged (EAT) 3      |
|        | AT1G61290   | 3     | syntaxin of plants 124                         |
| MIR319 | AT2G28056   | 2     | MIR172/MIR172A; miRNA                           |
|        | AT2G28550   | 2     | related to AP2.7                                |
|        | AT2G39250   | 3     | Integrase-type DNA-binding superfamily protein  |
|        | AT3G49690   | 3     | myb domain protein 84                          |
|        | AT3G5512    | 2     | Integrase-type DNA-binding superfamily protein  |
|        | AT3G5512    | 3     | MIR172D; miRNA                                  |
|        | AT5G04275   | 3     | MIR172/MIR172B; miRNA                           |
|        | AT5G9505    | 3     | MIR172E; miRNA                                  |
| MIR390 | AT2G38325   | 2     | MIR390A; miRNA                                  |
| MIR393 | AT3G62980   | 1     | F-box/RNI-like superfamily protein             |
|        | AT4G03190   | 2     | GRR1-like protein 1                            |
| MIR394 | AT1G27340   | 1     | Galactose oxidase/kelch repeat superfamily protein |
|        | AT3G29660   | 3     | transposable element gene                       |
|        | AT5G09670   | 3     | loricin-related                                |
|        | AT5G09672   | 3     | conserved peptide upstream open reading frame 21|
| MIR395 | AT1G69793   | 2     | MIR395A; miRNA                                  |
|        | AT1G69792   | 2     | MIR395D; miRNA                                  |
|        | AT1G69795   | 2     | MIR395E; miRNA                                  |
|        | AT1G26975   | 3     | MIR395B; miRNA                                  |
|        | AT1G26985   | 3     | MIR395C; miRNA                                  |
|        | AT1G69797   | 3     | MIR395F; miRNA                                  |
|        | AT2G41060   | 3     | RNA-binding (IRRM/RBD/RNP motifs) family protein |
|        | AT3G32280   | 3     | ATP-dependent helicase family protein           |
|        | AT4G14770   | 3     | TESMIN/TSO1-like CXC 2                         |
|        | AT5G04140   | 3     | glutamate synthase 1                           |
### Table 5. Cont.

| miRNA   | Target          | Score | Annotation                                                                 |
|---------|-----------------|-------|-----------------------------------------------------------------------------|
| AT5G19900 | PRLI-interacting factor, putative |
| AT2G28780 | unknown protein |       |
| AT1G50930 | unknown protein |       |
| AT5G43780 | Pseudouridine synthase/archaeosine transglycosylase-like family protein |
| AT3G22890 | ATP sulfurylase 1 |       |
| AT4G14680 | Pseudouridine synthase/archaeosine transglycosylase-like family protein |
| AT4G06509 | transposable element gene |       |
| AT5G13630 | magnesium-chelatase subunit chlH, chloroplast, putative/Mg-protoporphyrin IX chelatase |
| MIR396   | AT5G14130 Peroxidase superfamily protein |
| AT1G58020 | transposable element gene |       |
| AT2G45480 | growth-regulating factor 9 |       |
| AT3G33106 | transposable element gene |       |
| AT5G01370 | ALC-interacting factor 1 |       |
| AT5G07700 | myb domain protein 76 |       |
| AT5G35407 | MIR396B; miRNA |       |
| AT2G36400 | growth-regulating factor 3 |       |
| AT3G01910 | sulfite oxidase |       |
| AT2G04270 | RNAse E/G-like |       |
| AT3G52910 | growth-regulating factor 4 |       |
| AT5G16690 | origin recognition complex subunit 3 |       |
| MIR397   | AT5G18420 unknown protein |       |
| AT2G29130 | laccase 2 |       |
| AT4G05105 | MIR397A; miRNA |       |
| MIR398   | AT5G14550 Core-2/I-branching beta-1,6-N-acetylglucosaminyl transferase family protein |
| MIR403   | AT1G31280 Argonaute family protein |       |
| AT1G03060 | Beige/BEACH domain; WD domain, G-beta repeat protein |       |
| AT4G30620 | Uncharacterised BCR, YbaB family COG0718 |
| MIR408   | AT2G47020 Peptide chain release factor 1 |       |
| AT2G02850 | plantacyanin |       |
| MIR857   | AT1G4610 valyl-tRNA synthetase/valine–tRNA ligase (VALRS) |       |
| AT1G74060 | Ribosomal protein L6 family protein |       |
| AT5G28350 | Quinoprotein amine dehydrogenase |       |
| AT1G06670 | nuclear DEIH-boxhelicase |       |
| AT1G17040 | SH2 domain protein A |       |
| AT1G33475 | SNARe-like superfamily protein |       |
| AT1G43590 | transposable element gene |       |
| AT1G48010 | Plant invertase/pectin methylesterase inhibitor superfamily protein |       |
| AT3G20090 | cytochrome P450, family 705, subfamily A, polypeptide 18 |       |
| AT3G54230 | suppressor of abe3-5 |       |
| AT3G57660 | nuclear RNA polymerase A1 |       |
| miRNA | Target      | Score | Annotation                                                                 |
|-------|-------------|-------|-----------------------------------------------------------------------------|
|       | AT3G61480   | 3     | Quinoprotein amine dehydrogenase, beta chain-like; RIC1-like guanyl-nucleotide exchange factor |
|       | AT4G08460   | 3     | Protein of unknown function (DUF1644)                                      |
|       | AT4G10170   | 3     | SNAP-like superfamily protein                                               |
|       | AT5G06040   | 3     | self-incompatibility protein-related                                       |
|       | AT5G59470   | 3     | Mannose-P-dolichol utilization defect 1 protein                             |
| MIR858| AT1G06180   | 3     | myb domain protein 13                                                       |
|       | AT1G66230   | 3     | myb domain protein 20                                                       |
|       | AT2G47460   | 3     | myb domain protein 12                                                       |
|       | AT3G08500   | 3     | myb domain protein 83                                                       |
|       | AT4G12350   | 3     | myb domain protein 42                                                       |
|       | AT5G35550   | 3     | Duplicated homeodomain-like superfamily protein                             |
|       | AT5G49330   | 3     | myb domain protein 111                                                      |
| MIR1310| AT2G16592  | 3     | Bifunctional inhibitor/lipid-transfer protein/seed storage 25 albumin superfamily protein |
|       | AT3G29050   | 3     | receptor-like protein kinase-related                                        |
| MIR1448| AT5G40100  | 1     | Disease resistance protein (TIR-NBS-LRR class) family                      |
|       | AT1G65790   | 2     | receptor kinase 1                                                           |
|       | AT1G65800   | 2     | receptor kinase 2                                                           |
|       | AT3G46530   | 2     | NB-ARC domain-containing disease resistance protein                         |
|       | AT1G63350   | 3     | Disease resistance protein (CC-NBS-LRR class) family                       |
|       | AT2G30740   | 3     | Protein kinase superfamily protein                                          |
|       | AT4G00420   | 3     | Double-stranded RNA-binding domain (DsRBD)-containing protein              |
| MIR1511| AT3G28160  | 2     | transposable element gene                                                   |
|       | AT5G42820   | 2     | Zinc finger C-x8-C-x5-C-x3-H type family protein                           |
|       | AT1G03050   | 3     | ENTH/ANTH/VHS superfamily protein                                           |
|       | AT1G17340   | 3     | Phosphoinositide phosphatase family protein                                 |
|       | AT1G61610   | 3     | S-locus lectin protein kinase family protein                                |
|       | AT3G21360   | 3     | 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein    |
|       | AT4G24450   | 3     | phosphoglucon, water dikinase                                                |
|       | AT5G27965   | 3     | transposable element gene                                                   |
|       | AT5G32690   | 3     | Pseudogene of AT2G29880                                                     |
| MIR2111| AT3G27150   | 3     | Galactose oxidase/kelch repeat superfamily protein                         |
| MIR2592| AT4G11040   | 3     | Protein phosphatase 2C family protein                                      |
| MIR2916| AT1G15780   | 3     | unknown protein                                                             |
|       | AT1G50310   | 3     | sugar transporter 9                                                         |
|       | AT1G65875   | 3     | pseudogene                                                                  |
|       | AT1G66120   | 3     | AMP-dependent synthetase and ligase family protein                          |
|       | AT4G00450   | 3     | RNA polymerase II transcription mediators                                   |
|       | AT5G66710   | 3     | Protein kinase superfamily protein                                          |
| MIR4414| AT2G04860   | 3     | Tetratricopeptide repeat (TPR)-like superfamily protein                    |
| MIR4415| AT3G14490   | 3     | Terpenoid cyclases/Protein prenyltransferases superfamily protein           |
| MIRS139| AT3G28160   | 0     | transposable element gene                                                   |
|       | AT3G27965   | 1     | transposable element gene                                                   |
to detect and measure the expression levels of miRNAs in various tissues. The relative quantitative results demonstrated that all selected miRNAs were expressed in different organogenetic tissues and with entirely different expression profiles. On the basis of abundance trends at the six developmental stages, the 14 miRNAs were divided into four clusters through multivariate statistical analysis using general cluster analysis procedure of SPSS Statistics 18.0 (Figure 6).

The first cluster, comprising acr-miR159a, acr-miR319a, acr-miR162, acr-miR171q, acr-miR390a, and acr-miR396, exhibited similar expression patterns and showed a low expression level compared with the other three groups. These miRNAs were barely accumulated during stages S1 and S2 and were induced at stages S3 and S4 when the peak expression level was observed (Figure 7A–F), which implied that their accumulation may be required for embryogenic callus formation. The second cluster, consisting of acr-miR164a, acr-miR167a, and acr-miR168a, showed a stage-specific expression pattern. The expression level peaked during S4 but at the other stages expression was less abundant or undetectable (Figure 7G–I). The cultures were transferred to light at the S4 stage, which suggested these miRNAs might have an important relationship with light. In the third cluster, acr-miR156, acr-miR166, and acr-novel2* were expressed at varied levels in the different stages. Their major peak in expression was observed at S3, whereas expression at the other stages was relatively lower (Figure 7J–L). The striking differences in expression inferred their function is exerted in different developmental stages. The remaining two miRNAs (acr-miR397 and acr-miR398) showed relatively high expression levels in most of the detected tissues, especially acr-miR397. In this group, the miRNAs expression level rose continuously during the successive developmental stages except at S4, and accumulated in S3 and reached their highest levels in the adventitious shoots at S6 (Figure 7M–N), which suggested these miRNAs play a major regulatory role in morphogenesis during advanced differentiation of A. crassicarpa adventitious shoots.

The above results concluded that the different miRNAs were indicated to have functions at different developmental stages during A. crassicarpa organogenesis.

Discussion

In vitro organogenesis refers to the process involving regeneration of adventitious shoots, adventitious roots or other organs from plant tissue or cell aggregates (callus), which is an important aspect of plant development [45]. Many genes involved in the regulation of organogenesis have been investigated [46], but the molecular mechanisms underlying the process are still not well understood. Previous studies have confirmed that miRNAs play important roles in a variety of developmental processes. However, the role of miRNAs during organogenesis is poorly studied. In the present investigation, we used a high-throughput strategy to perform large-scale cloning and characterization of miRNAs involved in A. crassicarpa adventitious shoot organogenesis. We identified 189 known and 7 novel miRNAs from more than 57 miRNA families, and 14 conserved and novel miRNAs were selected for analysis of
expression patterns by qRT-PCR. These results provide valuable information on the molecular mechanism of organogenesis in *A. crassicarpa*. Furthermore, target gene prediction and GO annotation demonstrated that putative miRNA targets were involved in a broad variety of regulatory events, including molecular functions, biological processes and cellular components. According to specific stages, the expression patterns of 14 selected miRNAs were observed.

Micropropagation is an important and reliable technique for the production of large quantities of many plant species, particularly as a tool for large-scale plant breeding programs, and is used as a model system to research cell differentiation and development during organogenesis [47]. Changes in miRNA expression during plant somatic embryogenesis has been confirmed in many plants species, such as *A. thaliana* [48], rice [49], Japanese larch [50], Valencia sweet orange [51], Liriodendron chinense [52], loblolly pine [53], and longan [54]. However, the function of miRNAs during plant organogenesis is poorly studied. In order to better understand the potential modulation of miRNAs in organogenesis, we studied the expression patterns of miRNAs utilizing the *A. crassicarpa* organogenesis in *vitro* culture system described by Yao et al [18]. Combined with a bioinformatic analysis and comparison with miRNA families that are expressed during somatic embryogenesis in other plant species, the results revealed some of the important miRNAs functions related to plant regeneration. Differences in expression pattern were observed among 14 miRNAs analyzed by qRT-PCR. One group of miRNAs (*acr-miR171q, acr-miR319a, acr-miR162, and acr-miR171q*) were up-regulated at S3 and the expression level peaked at S4 (Figure 7). *MiR159* is a widespread gene present in many plant and animal species [55]. As a highly conserved miRNA in the plant kingdom, *miR159* negatively controls gene expression by targeting mainly MYB33 and MYB65 during seed germination and floral development [56–57]. In the present study, *acr-miR159a* transcripts accumulated until embryogenic callus was present at stage S3 of *A. crassicarpa* organogenesis, which suggested that *acr-miR159a* might modulate gene expression during embryogenic callus differentiation. This result is similar to the pattern observed in larch [50] and longan [54]. *Acr-miR319a*, which has an extremely similar sequence to that of the *miR159* family, showed similar relative expression levels during *A. crassicarpa* organogenesis [58]. *Acr-miR319a* and *acr-miR159*, showed the same putative target (MYB transcription factors) in the regulation of organogenesis in the present study, which suggests they play similar regulatory roles. The functions of *miR171* and *miR162* are rarely reported. In *A. thaliana*, the potential target of *miR171* is the scarecrow-like (*SCL*) gene family possessing the GRAS domain [59], which is consistent with our findings (Table 5). In our study, these two miRNAs were up-regulated at S3 and S4, indicating that *miR171* and *miR162* might function in the embryogenic callus, which is consistent with rice somatic embryogenesis [49]. Expression of the remaining miRNAs of the first cluster, *acr-miR590a* and *acr-miR596*, was elevated at S3 then declined, but was maintained at a relatively high level from S3 to S5. *MiR296* is reported to target growth-regulating factor (*GRF*) transcription factors, performing a negative coordinating role in leaf cell proliferation of *A. thaliana* [60]. Overall, members of the first cluster might play regulatory roles in the embryogenic callus stages of *A. crassicarpa* organogenesis.

S4 is a crucial stage during *A. crassicarpa* organogenesis when buds are induced and began to turn green, and are ready to develop into adventitious shoots. miRNAs of the second cluster, consisting of *acr-miR164a, acr-miR167a*, and *acr-miR168a*, exhibited stage-specific expression, which indicated that they may have stage-specific functions during bud formation. These three

### Table 6. miRNAs associated with plant development.

| MiRNA   | Target gene                      | Function                                  |
|---------|----------------------------------|-------------------------------------------|
| miR156  | SPL                              | Flowering control                         |
| miR159  | MYB                              | Floral initiation and anther development; seed germination |
| miR162  | DCLI                             | miRNA biogenesis                         |
| miR164  | CUC                              | Leaf development                         |
| miR166  | ClassIII HD-ZIP transcription factor | Shoot apical meristem and lateral organ formation |
| miR167  | ARF                              | Gynoecium and stamen development          |
| miR168  | AGO1                             | miRNA pathway regulation                  |
| miR171  | SCL                              | Flower development                       |
| miR319  | TCP transcription factors        | Morphogenesis of leaf                     |
| miR390  | TAS3 family of tasiRNA-generating transcripts | Developmental Time and Pattern |
| miR396  | GRF                              | Leaf development                         |
| miR397  | Laccases                         | Metabolism                               |
| miR398  | CSD and CytC oxidases/subunit V  | Stress response                          |

[53, 54]. As a highly conserved miRNA in the plant kingdom information on the molecular mechanism of organogenesis in *A. crassicarpa*. However, the function of miRNAs during organogenesis, plant organogenesis is poorly studied. In order to better understand the potential modulation of miRNAs in organogenesis, we studied the expression patterns of miRNAs utilizing the *A. crassicarpa* organogenesis in *vitro* culture system described by Yao et al [18]. Combined with a bioinformatic analysis and comparison with miRNA families that are expressed during somatic embryogenesis in other plant species, the results revealed some of the important miRNAs functions related to plant regeneration. Differences in expression pattern were observed among 14 miRNAs analyzed by qRT-PCR. One group of miRNAs (*acr-miR319a, acr-miR319b, acr-miR162, and acr-miR171q*) were up-regulated at S3 and the expression level peaked at S4 (Figure 7). *MiR159* is a widespread gene present in many plant and animal species [55]. As a highly conserved miRNA in the plant kingdom, *miR159* negatively controls gene expression by targeting mainly MYB33 and MYB65 during seed germination and floral development [56–57]. In the present study, *acr-miR159a* transcripts accumulated until embryogenic callus was present at stage S3 of *A. crassicarpa* organogenesis, which suggested that *acr-miR159a* might modulate gene expression during embryogenic callus differentiation. This result is similar to the pattern observed in larch [50] and longan [54]. *Acr-miR319a*, which has an extremely similar sequence to that of the *miR159* family, showed similar relative expression levels during *A. crassicarpa* organogenesis [58]. *Acr-miR319a* and *acr-miR159*, showed the same putative target (MYB transcription factors) in the regulation of organogenesis in the present study, which suggests they play similar regulatory roles. The functions of *miR171* and *miR162* are rarely reported. In *A. thaliana*, the potential target of *miR171* is the scarecrow-like (*SCL*) gene family possessing the GRAS domain [59], which is consistent with our findings (Table 5). In our study, these two miRNAs were up-regulated at S3 and S4, indicating that *miR171* and *miR162* might function in the embryogenic callus, which is consistent with rice somatic embryogenesis [49]. Expression of the remaining miRNAs of the first cluster, *acr-miR590a* and *acr-miR596*, was elevated at S3 then declined, but was maintained at a relatively high level from S3 to S5. *MiR296* is reported to target growth-regulating factor (*GRF*) transcription factors, performing a negative coordinating role in leaf cell proliferation of *A. thaliana* [60]. Overall, members of the first cluster might play regulatory roles in the embryogenic callus stages of *A. crassicarpa* organogenesis.

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| miR168  | AGO1                             | miRNA pathway regulation                  |
| miR171  | SCL                              | Flower development                       |
| miR319  | TCP transcription factors        | Morphogenesis of leaf                     |
| miR390  | TAS3 family of tasiRNA-generating transcripts | Developmental Time and Pattern |
| miR396  | GRF                              | Leaf development                         |
| miR397  | Laccases                         | Metabolism                               |
| miR398  | CSD and CytC oxidases/subunit V  | Stress response                          |

Component analysis of predicted targets for 37 differentially expressed miRNAs. (A) Molecular functions was divided into 10 functional groups. (B) Biological processes was divided into 14 functional groups. (C) Cellular components was divided into seven functional groups.
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**Figure 5. Functional classification of microRNA targets.** Gene ontology of the predicted targets for 37 differentially expressed miRNAs. (A) Molecular functions was divided into 10 functional groups. (B) Biological processes was divided into 14 functional groups. (C) Cellular components was divided into seven functional groups.
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miRNAs were expressed at low or undetectable levels at all stages except S4, during which expression peaked. In *Arabidopsis*, miR164 mainly controls the NAM/ATAF/CUC (NAC1) domain-transcription factor family. NAC1 is involved in transitions in auxin signalling, and facilitates growth of lateral roots [61]. CUC regulates organ separation from fasciculate buds during embryogenesis. In the present study, the same putative target (NAC-domain transcription factor) implied that miR164 has the same function in *A. crassicarpa*. MiR168 targets the AGO1 gene, which is involved in plant development by feedback regulation. The expression patterns of acr-miR164a and acr-miR168a were consistent with results reported for citrus after callus is cultured in the light [51]. However, our findings were inconsistent with the pattern observed in larch, in which miR168 was not expressed at a notably high level when callus began to turn green [50]. These conflicting results suggest that the function of miR168 might vary between species. Aux/IAA and ARF, the putative targets of miR167, are two important protein families that respond to auxin signal. Ru et al. [62] suggested that the high abundance of ARF8 led to the low expression level of miR167. These results imply the target of miR167 conservative property. In conclusion, this cluster of miRNAs may function in the bud formation stages and response to light in *A. crassicarpa* organogenesis.

The important events that occur during *A. crassicarpa* adventitious shoot organogenesis from a zygotic embryo involve redifferentiation. In rice [49], miR1356 is important in the transition from undifferentiated to differentiated callus during somatic embryogenesis by targeting SPL genes. Zhang et al. [50] and Wu et al. [51] reported that SPL controls the somatic embryo induction process. SPL genes also have other regulatory roles in different biological processes, such as induction of the floral transition and consequent shortening of the vegetative phase [63], and regulation of the juvenile to adult transition during plant development [64]. In the present study, acr-miR159a was accumulated at S3 while the zygotic embryo is differentiating into embryogenic callus during organogenesis. As shown in Figure 7, the abundance of the other members of the third cluster (acr-miR166h and acr-novel2*) also increased continuously until S3, but exhibited very low or undetectable expression levels during S4–S6 and especially at S5. Given that these three miRNAs showed similar expression patterns, we hypothesize that they modulate redifferentiation during induction of organogenesis in *A. crassicarpa* as the fatal genes.

With regard to the fourth cluster, relatively high expression of acr-miR397 and acr-miR398 was observed. MiR397, for which a target gene of laccase is implicated, is associated with lignin biosynthesis and primary cell wall [65–66]. The detection of miR397 and corresponding targets occurs during the regulation of lignification and thickening of the cell wall in secondary cell growth in rice, larch and citrus [49–51]. MiR398 is known to target Cu/Zn superoxide dismutases (CDSs), which are associated with stress response [43]. In the present study, the levels of acr-miR397 and acr-miR398 increase continuously until S6, at which stage the expression level peaked, except for a slight decline at S4. Zygotic embryos undergo differentiation at stages S1–S3 until the formation of buds at S4, which suggests that laccase, the target gene of miR397, regulates lignification and cell wall thickness during organogenesis. The decrease in expression level of miR397 at S4, when a cluster of adventitious shoots had developed, is attributed to negative modulation of the formation of thickened cell walls for adventitious shoots.

In summary, a global analysis of miRNAs expression during *A. crassicarpa* adventitious shoot organogenesis was carried out. The results of a bioinformatic analysis and experimental tests revealed putative regulatory functions for the miRNAs in *Acacia crassicarpa* organogenesis. These findings provide important information for deep sequencing research of miRNAs and future large-scale propagation and breeding of leguminous trees.
miRNAs during Organogenesis of Acacia crassicarpa

Figure 7. qRT-PCR analysis of relative expression levels of selected miRNAs at six stages of Acacia crassicarpa organogenesis. The fold change in gene expression was normalized to controls (mature zygotic embryo) with the 2^(-deltaCt) method using 5.8S rRNA as an internal standard. Templates for all miRNAs real-time PCR were 1/20 dilutions of original cDNAs reverse-transcribed from 300 ng miRNA. Each bar shows the mean of triplicate assays.
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Author Contributions
Conceived and designed the experiments: XX WL YJ. Performed the experiments: WL WY. Analyzed the data: WL XW LH. Contributed reagents/materials/analysis tools: FZ DL WW HY. Wrote the paper: WL WY.

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