Identification of the Conserved and Novel miRNAs in Mulberry by High-Throughput Sequencing

Ling Jia, Dayan Zhang, Xiwu Qi, Bi Ma, Zhonghuia Xiang, Ningjia He*

State Key Laboratory of Silkworm Genome Biology, Southwest University, Beibei, Chongqing, China

Abstract

miRNAs are a class of non-coding endogenous small RNAs. They play vital roles in plant growth, development, and response to biotic and abiotic stress by negatively regulating genes. Mulberry trees are economically important species with multiple uses. However, to date, little is known about mulberry miRNAs and their target genes. In the present study, three small mulberry RNA libraries were constructed and sequenced using high-throughput sequencing technology. Results showed 85 conserved miRNAs belonging to 31 miRNA families and 262 novel miRNAs at 371 loci. Quantitative real-time PCR (qRT-PCR) analysis confirmed the expression pattern of 9 conserved and 5 novel miRNAs in leaves, bark, and male flowers. A total of 332 potential target genes were predicted to be associated with these 113 novel miRNAs. These results provide a basis for further understanding of mulberry miRNAs and the biological processes in which they are involved.

Introduction

miRNAs, which are found in animals and plants, are a class of 19–24 nt non-coding small RNA molecules. They negatively regulate genes at the transcriptional and post-transcriptional level by cleaving the target mRNA and suppressing the translation of target mRNA [1]. miRNAs are encoded by MIR genes. In plants, MIR genes are transcribed into a pri-miRNA with a cap and a poly (A) tail and thereafter processed into a pre-miRNA, which is further cleaved into a miRNA/miRNA* duplex. The last nucleotide of the 3′ terminal in the duplex is methylated, and then the plant miRNA is loaded into the ARGONAUTE1 (AGO1) complex leading to the cleavage of the target mRNA [1]. Increasing amounts of evidence have demonstrated that miRNAs play crucial roles in plant growth, development, and response to biotic and abiotic stress [2–4]. For example, miR156 regulates the transition of juvenile to adult in Arabidopsis thaliana [5]. miR172 negatively regulates the cell fate specification in flower development of A. thaliana [6]. miR160 controls the formation of root caps by targeting auxin response factors ARF10 and ARF16, both of which restrict the stem cell niche and promote columella cell differentiation [7].

Despite the importance of miRNA, the first miRNA, lin-4, was discovered until 1993 from Caenorhabditis elegans [8]. In 2001, tens of miRNAs were identified in several animal species by directly cloning and sequencing [9,10]. Since then, bioinformatic prediction and cloning have been used to identify many miRNAs in animals and plants [11–15]. However, although predictions that rely on the sequence can predict the conserved miRNAs easily, it is difficult to identify species-specific miRNAs. The cloning method can only be used to identify small-scale miRNAs. In 2005, high-throughput sequencing technology was first used to sequence the small RNA libraries of A. thaliana and many miRNAs were identified [16]. This next-generation sequencing technology enables massive sequencing and detection of minimally abundant small RNA. It has become the method of choice for sequencing of the genome, transcriptome, and small RNA transcriptome [17–21]. A recent analysis of miRNA (based on Release 19, http://www.mirbase.org/, August 2012) showed a total of 21,264 miRNAs to be registered in miRBASE. This number is almost 20 times than that in Release 6.0 (Release 6.0, ftp://mirbase.org/pub/mirbase/CURRENT/README, April 2005). The expansion of currently available miRNA information can be attributed to the development of this technique.

Mulberry trees are widely planted in Europe, Africa, Asia, and the United States. This tree belongs to the genus Morus, family Moraceae, order Rosales [22,23]. Mulberry leaves have been used to feed silkworms for silk production for about 5,000 years. In addition to being the sole nutritional source of the silkworm, mulberry tree have many other multiple uses. In particular, the secondary metabolites of mulberry plants are widely used as medicines [24–26]. In the current genomic era, the genome data are an important resource for gene identification and characterization. The completion of the mulberry genome has allowed scholars to look at mulberry genes comprehensively [18]. Deep sequencing of transcripts can reveal many important gene products, such as miRNAs, which have been shown to be crucial to plant development and stress responses [2–4]. For this reason, mulberry miRNAs were sequenced and analyzed in the present study. Three small RNA libraries of mulberry tissues (leaves, bark, and male flowers) were constructed and used for sequencing. Conserved, novel mulberry’s miRNAs and their target genes were identified. The expression profiles of 9 conserved and 5 novel miRNAs were analyzed by qRT-PCR. Furthermore, the target genes of these miRNAs were predicted by bioinformatic analysis. The predicted target genes were further confirmed by qRT-PCR.

Citation: Jia L, Zhang D, Qi X, Ma B, Xiang Z, et al. (2014) Identification of the Conserved and Novel miRNAs in Mulberry by High-Throughput Sequencing. PLoS ONE 9(8): e104409. doi:10.1371/journal.pone.0104409

Editor: Jinfa Zhang, New Mexico State University, United States of America

Received February 20, 2014; Accepted July 8, 2014; Published August 13, 2014

Copyright: © 2014 Jia 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 project was funded by the research grants from the National Hi-Tech Research and Development Program of China (No. 2013AA100605-3), the “111” Project (B12006), the Science Fund for Distinguished Young Scholars of Chongqing (Grant No. cstc2011jjqj0010), and Chong Qing Science & Technology Commission (NO. cstc2012jyx00001). 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.

* Email: hejia@swu.edu.cn

References:

1. Lee RM, Feinbaum RL, Ambros V (1993) hairpin RNA that directs posttranscriptional gene silencing in Caenorhabditis elegans. Cell 75: 843–854
2. Sontheimer EJ, Mertens F, Walter P (2009) RNAi in plants. Cell 137: 165–175
3. Ding X, Peng X, Yan L, Wang Z, Liu Y, et al. (2007) Identification of differentially expressed miRNAs in rice. Proc Natl Acad Sci USA 104: 7287–7292
4. Hu W, Sun X, Yang H, Fan Y, Liu L, et al. (2007) Identification of differentially expressed miRNAs in rice leaves infected with rice blast fungus. Plant Cell Environ 30: 1399–1409
5. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, et al. (2005) An international code for secretion and nomenclature of RNA-binding proteins. Nat Biotechnol 23: 545–546
6. Liu Y, Zhu J, Zhao W, Li L, Wang Z, et al. (2007) Identification of miRNAs in rice. Curr Biol 17: 1933–1938
7. Liu Y, Zhu J, Zhao W, Li L, Wang Z, et al. (2007) Identification of miRNAs in rice. Curr Biol 17: 1933–1938
mulberry miRNAs were confirmed in three tissues using stem-loop quantitative real-time PCR. These results expand our knowledge of the diversity and specificity of mulberry miRNAs and provide a basis for further understanding of the biological mechanisms that take place in mulberry plants.

Materials and Methods

Plant material and construction of small RNA library

The wild mulberry species *Morus notabilis* grows in a pristine forest in Yaan, Sichuan Province, in southwest of China. This tree is located at 29°45.278' north latitude, 102°53.878' east longitude. It is a mulberry species used for the genome sequencing. Young leaves, bark and male flowers from *M. notabilis* were collected in the spring of 2013. The samples were immediately frozen in liquid nitrogen and stored in −80°C. No specific permissions were required for these activities. The field studies did not involve endangered or protected species. Small RNA libraries of *M. notabilis* were constructed as described elsewhere with a few modifications [27]. Briefly, the total RNA of three *M. notabilis* tissues was extracted using RNAiso plus (D9108A, Takara, China) in accordance with the manufacturers’ instruction. Samples were then subjected to 15% denaturing polyacrylamide gel electrophoresis (PAGE). Small RNA fragments of 18–30 nt were separated, purified, and ligated with the 5’, 3’adapter sequentially. After reverse transcription and PCR, about 20 μg products of three tissues were separately sequenced using Illumina HiSeq-2000 (BGI-Shenzhen, China).

Bioinformatics analysis of small RNA

Raw data were filtered using a Perl script to delete low-quality reads, chip adapter sequences, and contaminations. The sequences ≥18 nt of clean data were annotated in the Rfam database (Release 10.1) [http://www.sanger.ac.uk/software/Rfam] and Genbank non-coding RNA database [http://www.ncbi.nlm.nih.gov/] to remove non-coding RNA (tRNA, tRNA, snRNA, snoRNA) and degradation fragments of miRNA. The remaining sequences were aligned against miRNA database, miRBASE (Release 19) [http://www.mirbase.org/], and perfectly matched sequences were considered conserved *M. notabilis* miRNAs.

Prediction of novel miRNAs

The unannotated small RNAs 18–30 nt in length were searched against *M. notabilis* genome. Novel *M. notabilis* miRNA were predicted using Mireap software [http://sourceforge.net/projects/ mireap/] with modifications basing on the default parameters: (1) the miRNA sequence length was 18–25 nt; (2) the miRNA reference sequence length was 20–23 nt; (3) the maximum copy number of miRNAs in any of the previous studies was 20; (4) the free energy of miRNA precursor was less than −18 kcal/mol; (5) the maximum space between miRNA and miRNA* was 300 nt; (6) the minimal base pairs of miRNA and miRNA* was 16; (7) the maximum bulge of miRNA and miRNA* was 4; (8) the maximum asymmetry of miRNA/miRNA* duplex was 4; and (9) the flank sequence length of miRNA precursor was 20 nt. Hairpin structures of potential novel miRNA precursors were checked manually. The criteria were as follows: (1) the minimal folding free energy index (MFEI) of potential novel miRNA precursor was required be at least 0.85 [28]; (2) the asymmetric bulges of miRNA/miRNA* duplex were less than 3; (3) there were fewer than 4 mismatches between miRNA and miRNA*; (4) if the miRNA sequence did not fit these criteria, but corresponding miRNA* were detected, miRNA were also considered potentially novel miRNA.

Expression of mulberry miRNAs and target genes as assessed using qRT-PCR

Fourteen miRNAs were chosen for stem-loop RT-PCR in three mulberry tissues as previous described [29]. Briefly, each 1 μg total RNA was hybridized with a miRNA-specific stem-loop primer (10 pmol). The hybridized miRNA molecules were reverse transcribed to cDNA in 10 μL reaction using Reverse Transcriptase M-MLV (2641A, TaKaRa, China) in accordance with the manufacturer’s instruction. The resultant was then diluted three-fold and 1.5 μL cDNA was used as the template to perform the stem-loop RT-PCR with each miRNA specific forward primer and universal primer, as listed in Table S7. The reverse transcriptions for target genes were performed as follows. One microgram of total RNAs were reverse transcribed a 20 μL reaction using PrimerScript RT Reagent kit with gDNA Eraser (RR047A, TaKaRa, China) basing on the handbook described. The resultant was then diluted four-fold and 1 μL cDNA was used as the template to perform the RT-PCR with each target gene primers, as listed in Table S8. The PCR reactions were performed in ABI Step One Plus (Applied Biosystems, USA) using SYBR Premix Ex Taq II (RR820A, TaKaRa, China) as the following conditions: 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 30 s. The 5.8S rRNA and ribosomal protein L15 gene were used as inner controls. All reactions were assayed in triplicated. The relative expression level of miRNA was calculated using 2−ΔΔCT method.

Prediction of miRNA targets

miRNA target prediction was performed by aligning miRNAs with the *M. notabilis* genes using a perl script, which was designed to predict the miRNA targets according to the criteria described by Allen and Schwab [30] [31]. These criteria were as follows: (1) There had to be no more than 4 mismatches in the miRNA/target duplex. (G–U pairs were considered 0.5 of a mismatch). (2) There had to be no more than 2 adjacent mismatches in the miRNA/target duplex. (3) There had to be no adjacent mismatches at position 2–12 at the miRNA 5’ terminal of the miRNA/target duplex. (4) There had to be no mismatches in the position 10 and 11 of the miRNA/target duplex. (5) There had to be no more than 2.5 mismatches in the position 1–12 during the miRNA/target duplex. (6) The minimal free energy of miRNA/target duplex had to exceed 75% that of the miRNA when bound to its perfect complement.

Results

Small RNA in three mulberry tissues

In order to identify the miRNAs in mulberry plants, total RNA (integrity ≥8.0) was extracted from three mulberry tissues and three small RNA libraries were constructed for sequencing. These data have been deposited in NCBI/SRA database under accession number of SRP032829. A total of 11,752,747 reads (leaf), 11,491,921 reads (bark), and 10,513,612 reads (male flower) were obtained by sequencing, as shown in Table 1. After removing low quality sequences, adapters, and contaminated sequences, there were 10,992,174 (93.97%) clean reads ≥18 nt in size from leaves, 11,273,911 (98.55%) from bark, and 10,134,148 (96.83%) from male flowers. Among the clean reads ≥18 nt in size, the reads of miRNAs was 832,571 (leaf), 1,130,016 (bark), 2,359,403 (male flower). The total reads of three tissues were subjected to analyze the size distribution as shown in Figure 1. The 21–24 nt small RNAs made up 77.87%, 79.78%, and 81.39% of the reads from mulberry leaves, bark, and male flowers accounted, respectively. The most common size of small RNAs in leaves and bark was 24 nt, accounting for 38.75%
and 36.04% of the total, respectively. This was consistent with the size distribution patterns of small RNAs in *Arachis hypogaea* and *Raphanus sativus* [32,33]. However, the size distribution pattern of male mulberry flowers was different. The most common size for small RNAs in male flower tissue was 21 nt, which was consistent with *Fragaria × ananassa* and *Pinus contorta* [34,35].

**Conserved miRNAs in mulberry plants**

In order to investigate the conserved miRNAs in mulberry plants, unique small RNAs from three mulberry tissues were aligned against miRNAs registered in the miRBase database (Release 19). Using the principle of sequence perfect matching, the present analysis identified 85 conserved miRNAs belonging to 31 families, as shown in Table 2. There were 77, 70, and 70 miRNAs identified in leaf, bark, and male flower tissue, respectively. Among the 85 conserved miRNAs, 57 were common to all three tissue libraries (Figure S1B). The length distribution of 85 conserved miRNAs is shown in Figure S1A. A peak appeared at 21 nt (84.7%).

In a broader evolutionary context, mulberry miRNAs were compared to those of seven other plants, including five dicotyledons (*A. thaliana*, *Glycine max*, *Malus domestica*, *Populus trichocarpa*, *Ricinus communis*) and two monocotyledons (*Oryza sativa* and *Zea mays*). Of the 31 mulberry miRNA families, 24 were conserved in the seven plant species. These miRNA were classified into well-conserved miRNA families. Prominent among them were mulberry miR160b, miR164a, miR167a, miR169a, miR390, and miR396b, which completely matched their counterparts in the seven other plant species, suggesting those miRNAs were extremely conserved, and might play critical physiological roles in both dicotyledons and monocotyledons. However, 7 miRNA families, miR482, miR529, miR827, miR4995, and miR5523, were found in only one or two plant species (Table 2).

It has been reported that the sequencing frequency in Illumina technology was used to estimate the relative levels of expression of miRNAs [32]. The present data indicated that the conserved miRNA families were expressed across a vast range, from over 10,000 reads to fewer than 10 reads in mulberry, as shown in Table 2. Of the 31 miRNA families, the reads of miR156, miR166, miR167, miR168, and miR535 exceeded 10,000 in all three tissues. Eleven miRNA families (miR159, miR160, miR164, miR169, miR171, miR172, miR390, miR396, miR397, miR529 and miR4376) had more than 1000 reads at least in one tissue. Seven miRNA families (miR162, miR393, miR395, miR398, miR399, miR408 and miR4414) had numbers of sequence reads ranging from 100–1000 at least in one tissue, and the remaining miRNA families (miR319, miR492, miR827, miR828, miR858, miR2111, miR4995 and miR5523) had fewer than 100 reads in all three tissues.

**Novel miRNAs in mulberry plants**

One of the greatest advantages of high-throughput sequencing is that this technology can be used to discover species-specific miRNAs. Here, mireap software was used with several criteria to identify the novel mulberry miRNAs, as described in method section. Using un-annotated sequences from mulberry three tissues, 262 novel miRNAs in 371 mulberry genome loci were identified, as shown in Table S1. Previous studies have reported that miRNA* sequence can be used as criteria for the identification of novel miRNAs. In the present study, 90 miRNA* sequences were discovered in the precursors of novel miRNAs. This suggested that those 90 novel miRNAs must be real mulberry miRNAs. Most of the 90 miRNA* had much lower abundance than their partial strand miRNAs. However, several miR-
Identification of tissue-biased miRNAs

Some miRNAs were expressed in a tissue-specific/biased manner. Documentation of such miRNAs has served as foundational information for functional studies. In the present study, tissue-biased miRNA families were investigated by normalizing the reads in different data sets. miRNAs were considered tissue-biased if there were twice as many normalized reads in one tissue than in the other two tissues. As shown in Figure 2 and Table S2, 5 leaf-biased miRNA families (miR4995, miR827, miR828, miR172, and miR390), 5 bark-biased miRNA families (miR535, miR396, miR393, miR395, and miR319), and 8 male flower-biased miRNA families (miR156, miR529, miR160, miR397, miR398, miR108, miR169, and miR167) were identified in conserved mulberry miRNA families. Among mulberry novel miRNAs, 58 leaf-biased, 84 bark-biased, and 72 male flower-biased miRNAs were identified, as shown in Table S3. Among these tissue-biased novel miRNAs, 8, 6, and 15 novel miRNAs were only observed in leaf, bark, and male flower tissue, respectively (Table S4). These 29 novel miRNAs had very few reads, ranging in number from 5–136.

Detection of the expression of miRNAs using RT-PCR

To confirm the expression pattern of the mulberry miRNAs, 9 conserved and 5 novel miRNAs with different expression profiles were randomly selected for stem-loop RT-PCR analysis. As illustrated in Figure 3, miR827, miRn247, and miRn184 were more abundantly expressed in leaves than in bark or male flowers. miR396 and miRn62 were highly expressed in bark. Five mulberry miRNAs, miR156, miR160, miR169, miRn74, and miRn188, were expressed predominantly in male flowers. The remaining 4 non-tissue-biased conserved miRNAs (miR139, miR162, miR164, and miR168) exhibited expression pattern nearly identical to the results of the analysis of sequencing data. Taken together, the results of stem-loop RT-PCR were consistent with the expression pattern of the tissue-biased miRNAs identified using high-throughput sequencing.

Prediction of miRNA targets

Plant miRNAs play important roles in biological processes by cleaving target mRNAs and suppressing the translation of target genes. In order to understand the biological functions of mulberry miRNAs, the target genes of 31 mulberry conserved miRNAs representing 31 families with high reads and 262 novel miRNAs were predicted using the methods described above. As listed in Table S3 and Table S6, 89 target genes for 20 conserved miRNAs and 332 target genes for 113 novel miRNAs were annotated using the nr database. The majority of the target genes of conserved miRNAs were transcriptional factors, and as many miRNA targets were found to be conserved in mulberry plants as other plant species. These include miRNA-target pair associated with flower development, miR156-squamosa promoter-binding-like protein (SPL), miR159-MYB, miR166-homeobox-leucine zipper protein (HD-ZIP III), miR172-floral homeotic protein APETALA and partners associated with root development, miR164-NAC domain-containing protein and miR167-auxin response factor 6 (ARF). Functional proteins were also identified as targets of conserved miRNA including mno-miR397 (laccase), mno-miR395 (sulfate transporter), and mno-miR390 (receptor-like protein kinase). The

Table 1. Classification of small RNAs in the mulberry leaves, bark, and male flowers.

| Category                  | Leaves | Bark | Male flowers |
|---------------------------|--------|------|--------------|
| Unique reads              |        |      |              |
| Total reads               |        |      |              |
| Raw data                  | 11,752,747 | 11,491,921 | 10,513,612    |
| High quality              | 11,697,570 | 11,439,285 | 10,465,855    |
| Clean reads [size >= 18 nt] | 3,383,828 | 10,992,174 | 11,439,285    |
| Match genome build2       | 2,619,858 | 9,394,886 | 2,776,320     |
| Exon sense                | 75,103  | 195,404 | 77,783       |
| Exon antisense            | 175,011 | 401,988 | 151,119      |
| Intron antisense          | 140,785 | 298,027 | 153,833      |
| miRNAs                    | 26,956  | 832,571 | 31,463       |
| rRNA                      | 114,561 | 2,157,926 | 162,589      |
| Repeat                    | 250,148 | 525,805 | 223,690      |
| snoRNA                    | 2,882   | 13,281 | 4,210        |
| snRNA                     | 1,058   | 3,169  | 1,148        |
| tRNA                      | 12,054  | 368,395 | 20,001       |
| Unannotated small RNA     | 2,362,604 | 5,329,653 | 2,786,819    |
| Family | Name     | Reference miRNA | Sequence (5’-3’) | Length | Reads in leaf | Reads in bark | Reads in male flower | Ath | Gma | Mdm | Ptc | Rco | Osa | Zma |
|--------|----------|-----------------|------------------|--------|---------------|---------------|---------------------|-----|-----|-----|-----|-----|-----|-----|
| mIR156 | mno-mIR156a | zmaw-mIR156a-3p | GCTACCTCTCTCTCTGTCAGC | 22     | 0             | 46            | 4                  | -   | -   | -   | -   | ++  | ++  | ++  |
| mIR156 | mno-mIR156b | gma-mIR156b     | TTGACAGAAAGAGAGAGCACACA | 22     | 6             | 2             | 337                | +   | ++  | +   | +   | +   | +   | +   |
| mIR156 | mno-mIR156c | gma-mIR156c     | TTGACAGAAAGAGAGAGCACACAC | 21     | 12,787        | 117,270       | 768,488            | ++  | ++  | ++  | ++  | +   | +   | +   |
| mIR156 | mno-mIR156d | gma-mIR156d     | TTGACAGAAAGAGAGAGCACACAC | 21     | 2,942         | 14,574        | 8,723              | -   | ++  | ++  | +   | +   | +   | +   |
| mIR156 | mno-mIR156e | mdm-mIR156e     | TTGACAGAAAGAGAGAGCACACAC | 21     | 2,099         | 1,277         | 155,199            | +   | ++  | ++  | +   | +   | +   | +   |
| mIR156 | mno-mIR156f | ssl-mIR156      | TGACAGAAAGAGAGAGCACACAC | 21     | 22            | 378            | 65                 | -   | +   | +   | ++  | +   | -   | -   |
| mIR156 | mno-mIR156g | tcc-mIR156a     | TGACAGAAAGAGAGAGCACACAC | 21     | 8             | 4              | 1,198              | +   | ++  | +   | +   | +   | +   | +   |
| mIR159 | mno-mIR159a | ssp-mIR159a     | TGACAGAAAGAGAGAGCACACAC | 21     | 324           | 4,571          | 3,146              | +   | +   | +   | ++  | +   | +   | +   |
| mIR159 | mno-mIR159b | aly-mIR159b-3p  | TTTGTGTAAGGGAGTCTTC | 21     | 21            | 29             | 0                  | ++  | +   | +   | +   | +   | +   | +   |
| mIR160 | mno-mIR160a | aly-mIR160a-3p  | GCTGATGACGGCCGATGCA | 21     | 596           | 428            | 2,345              | -   | +   | -   | -   | -   | -   | -   |
| mIR160 | mno-mIR160b | cme-mIR160b     | TGCTTGGCTCCCTGATGCA | 21     | 11            | 14             | 13                 | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| mIR162 | mno-mIR162a | cme-mIR162a     | TGCTTGGCTCCCTGATGCA | 21     | 162           | 956            | 727                | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| mIR164 | mno-mIR164a | cme-mIR164a     | TGCTTGGCTCCCTGATGCA | 21     | 1,734         | 6,078          | 1,191              | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| mIR164 | mno-mIR164b | cme-mIR164b     | TGCTTGGCTCCCTGATGCA | 21     | 21            | 0              | 12                 | +   | +   | +   | +   | +   | +   | +   |
| mIR166 | mno-mIR166a | hbr-mIR166a     | TGCTTGGCTCCCTGATGCA | 22     | 26            | 51             | 27                 | -   | +   | +   | +   | -   | -   | -   |
| mIR166 | mno-mIR166b | cme-mIR166b     | TGCTTGGCTCCCTGATGCA | 21     | 198,685       | 238,486        | 252,806            | -   | ++  | ++  | ++  | -   | -   | -   |
| mIR166 | mno-mIR166c | gma-mIR166c     | TCGGACAGAGCCGATGCA | 21     | 20,100        | 168,078        | 39,124             | -   | ++  | +   | -   | -   | -   | -   |
| mIR166 | mno-mIR166d | gma-mIR166d     | GGAATGTTGGCTGGCTGGG | 21     | 3,935         | 975            | 901                | -   | +   | -   | -   | -   | ++  | ++  |
| mIR166 | mno-mIR166e | oea-mIR166e     | GGAATGTTGGCTGGCTGGG | 21     | 21            | 0              | 4                  | -   | -   | -   | -   | -   | ++  | ++  |
| mIR166 | mno-mIR166f | bdi-mIR166f     | GGAATGTTGGCTGGCTGGG | 21     | 31            | 56             | 194                | -   | +   | -   | -   | -   | -   | -   |
| mIR167 | mno-mIR167a | cme-mIR167a     | TGAACTGCGAAGATGCTTTAA | 22     | 574           | 84             | 1,526              | +   | +   | +   | +   | +   | +   | +   |
| mIR167 | mno-mIR167b | bna-mIR167b     | TGAACTGCGAAGATGCTTTAA | 22     | 117           | 125            | 28                 | ++  | +   | +   | +   | +   | +   | +   |
| mIR167 | mno-mIR167c | nta-mIR167c     | TGAACTGCGAAGATGCTTTAA | 22     | 75,303        | 12,129         | 140,989            | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| mIR167 | mno-mIR167d | ath-mIR167d     | TGAACTGCGAAGATGCTTTAA | 21     | 3,722         | 324            | 4,334              | -   | ++  | ++  | +   | +   | +   | +   |
| mIR168 | mno-mIR168a | aau-mIR168a     | GATCCCGCCTTCAGCATGCTTA | 24     | 9             | 27             | 12                 | -   | -   | +   | +   | -   | +   | +   |
| mIR168 | mno-mIR168b | mdm-mIR168b     | GATCCCGCCTTCAGCATGCTTA | 21     | 18,988        | 33,236         | 31,342             | ++  | ++  | ++  | ++  | +   | +   | +   |
| mIR169 | mno-mIR169a | cme-mIR169a     | CAGCGGCAGAATCGCCGG | 21     | 113           | 386            | 1314               | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| mIR169 | mno-mIR169b | nta-mIR169b     | CAGCGGCAGAATCGCCGG | 21     | 73            | 0              | 106                | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| mIR169 | mno-mIR169c | ntz-mIR169c     | CAGCGGCAGAATCGCCGG | 21     | 1                | 0              | 0                  | -   | +   | +   | -   | -   | -   | -   |
| mIR171 | mno-mIR171a | bna-mIR171a     | TGGATGAGAGCAGTGGCAAATC | 21     | 379           | 5              | 528                | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| mIR171 | mno-mIR171b | cme-mIR171b     | TGGATGAGAGCAGTGGCAAATC | 21     | 36            | 9              | 3                  | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| Family     | Name          | Reference miRNA | Sequence (5’-3’)             | Length | Reads in leaf | Reads in bark | Reads in male flower | Ath | Gma | Mdm | Ptc | Rco | Osa | Zma |
|------------|---------------|-----------------|------------------------------|--------|---------------|---------------|----------------------|-----|-----|-----|-----|-----|-----|-----|
| mno-miR171b | gma-miR171b-3p | CGAGCCGAATCATAATCCTC | 21  | 34  | 603  | 127           | -             | ++       | +   | +   | +   | +   | +   |
| mno-miR171a | mdm-miR171a-3p | TTGAGCCGTCACATGTCCTC | 21  | 32  | 1    | 0             | +           | ++       | ++  | ++  | +   | -   | +   |
| mno-miR171c | ptc-miR171a-3p | AGATTGAGCCGCGCAATTCCTC | 21  | 29  | 75   | 27            | +           | +        | ++  | ++  | +   | +   | +   |
| mno-miR171f | gma-miR171f-3p | TATTTGCUCTGACATTGTCCTC | 21  | 6   | 0    | 2             | -           | -        | -   | -   | +   | +   | +   |
| mno-miR171g | mdm-miR171g-3p | TTGAGGCCGTCACATGTCCTC | 21  | 5   | 600  | 8             | -           | +        | ++  | -   | -   | +   | +   |
| mno-miR171h | cme-miR171h-3p | TTGAGCCGTCACATGTCCTC | 21  | 4   | 0    | 5             | ++       | ++       | ++  | ++  | +   | -   | +   |
| mno-miR171i | gma-miR171i-3p | CGATTTGAGCCGCGCAATTCCTC | 21  | 2   | 0    | 0             | -           | -        | -   | -   | +   | +   | +   |
| mno-miR171j | gma-miR171j-3p | CGATTTGAGCCGCGCAATTCCTC | 21  | 19,265 | 8,484  | 1,245       | ++       | ++       | ++  | ++  | +   | -   | +   |
| mno-miR171k | gma-miR171k-3p | CGATTTGAGCCGCGCAATTCCTC | 21  | 143 | 13    | 13            | ++       | ++       | ++  | +   | +   | +   | +   |
| mno-miR171l | gma-miR171l-3p | CGATTTGAGCCGCGCAATTCCTC | 21  | 15  | 266  | 6             | ++       | ++       | ++  | ++  | +   | +   | +   |
| mno-miR171m | gma-miR171m-3p | CGATTTGAGCCGCGCAATTCCTC | 21  | 11 | 6    | 4             | -           | -        | ++  | -   | -   | +   | +   |
| mno-miR171n | gma-miR171n-3p | CGATTTGAGCCGCGCAATTCCTC | 21  | 3   | 2    | 0             | +           | +        | +   | +   | +   | +   | +   |
| mno-miR171o | gma-miR171o-3p | CGATTTGAGCCGCGCAATTCCTC | 21  | 1  | 0    | 0             | -           | +        | +   | -   | -   | +   | +   |
| mno-miR171p | gma-miR171p-3p | CGATTTGAGCCGCGCAATTCCTC | 21  | 43  | 104  | 47            | ++       | ++       | ++  | +   | +   | +   | +   |
| mno-miR171q | gma-miR171q-3p | CGATTTGAGCCGCGCAATTCCTC | 21  | 211 | 154  | 1,483        | ++       | ++       | ++  | +   | +   | +   | +   |
| mno-miR171r | gma-miR171r-3p | CGATTTGAGCCGCGCAATTCCTC | 21  | 21  | 0    | 1             | 0           | +        | +   | +   | +   | ++  | +   |
| mno-miR171s | gma-miR171s-3p | CGATTTGAGCCGCGCAATTCCTC | 21  | 111 | 1    | 83            | ++       | ++       | ++  | +   | +   | +   | +   |
| mno-miR171t | gma-miR171t-3p | CGATTTGAGCCGCGCAATTCCTC | 21  | 36  | 0    | 16            | -           | -        | -   | -   | +   | +   | +   |
| mno-miR171u | gma-miR171u-3p | CGATTTGAGCCGCGCAATTCCTC | 21  | 26  | 4    | 71            | +           | +        | +   | ++  | +   | +   | +   |
| mno-miR171v | gma-miR171v-3p | CGATTTGAGCCGCGCAATTCCTC | 21  | 11  | 0    | 0             | ++       | +        | +   | +   | +   | +   | +   |
| mno-miR171w | gma-miR171w-3p | CGATTTGAGCCGCGCAATTCCTC | 21  | 9   | 2    | 28            | ++       | +        | +   | +   | +   | +   | +   |
| Family | Reference miRNA | Sequence (5'-3') | Length | Reads in leaf | Reads in bark | Reads in male flower | Ath | Gma | Mdm | Ptc | Rco | Osa | Zma |
|--------|----------------|-----------------|--------|--------------|--------------|---------------------|-----|-----|-----|-----|-----|-----|-----|
| miR408 | mno-miR408a    | TGCCTCCTGCTCGCCGTCT | 21     | 0             | 0             | 2                   | +   | +   | +   | +   | +   | +   |
|        | mno-miR408b    | nta-miR408      | TGCTGTGCTCTCCCTGCCTG | 22     | 2             | 0             | 6                   | +   | +   | +   | +   | +   | +   |
|        | cme-miR408c    | ATGCACTGCACTTTCCCTGCC | 21     | 219           | 59            | 838                 | ++  | ++  | ++  | ++  | ++  | ++  |
| miR482 | mno-miR482b    | GAGGGCTGCTTTGGGAAGAAG | 22     | 23            | 54            | 25                  | -   | -   | +   | -   | -   | -   |
| miR529 | mno-miR529a    | AGAAGAGAGAGAGAGAGAAGAAG | 21     | 2851          | 367           | 6,790               | -   | -   | -   | -   | ++  | +   |
|        | cme-miR529b    | AGAAGAGAGAGAGCGAGCGC | 21     | 3             | 0             | 5                   | -   | -   | -   | -   | +   | +   |
| miR535 | mno-miR535a    | TGAACAGACAGACAGACAGACGC | 21     | 21,566        | 57,272        | 19,329              | -   | -   | ++  | -   | ++  | -   |
| miR827 | mno-miR827b    | TTAGTGACACATCACACGAAACA | 21     | 2             | 0             | 0                   | +   | -   | ++  | +   | +   | +   |
| miR828 | mno-miR828b    | TCTTGCTAATTGATATTCA | 22     | 5             | 1             | 0                   | +   | ++  | ++  | ++  | -   | -   |
| miR858 | mno-miR858b    | ATGCTGCACCTCTGTGACCTTG | 21     | 17            | 17            | 2                   | ++  | -   | +   | -   | -   | -   |
| miR211  | mno-miR2111b   | TATACTGACACCTGAGGTTTA | 21     | 11            | 10            | 10                  | ++  | ++  | ++  | +   | -   | -   |
| miR4376 | mno-miR4376b  | TACGGAGAGAGATAGCGCTGT | 22     | 9,353         | 1,231         | 6,682               | -   | ++  | +   | -   | -   | -   |
| miR4414 | mno-miR4414b  | AGCTGCTGACTGTTGACCTCA | 21     | 121           | 92            | 6                   | -   | +   | -   | -   | -   | -   |
| miR4995 | mno-miR4995b  | AGGCAGTGGCTTGTTAAGGG | 21     | 10            | 0             | 0                   | -   | ++  | -   | -   | -   | -   |
| miR5523 | mno-miR5523a  | TGGAGGACATATTTACTAG | 22     | 0             | 1             | 2                   | -   | -   | -   | -   | +   | -   |

"++" represents the mulberry miRNAs that are perfectly matched to those of other plant species. "+" represents mulberry miRNAs with 1–3 mismatches relative to the seven other plant miRNAs. "-" represents mulberry miRNAs with more than 3 mismatches relative to those of seven other plant species. ath, gma, mdm, ptc, rco, osa, and mno indicate Arabidopsis thaliana, Glycine max, Malus domestica, Populus trichocarpa, Ricinus communis, Oryza sativa, Zea mays, and Morus notabilis, respectively.

doi:10.1371/journal.pone.0104409.t002
targets of novel miRNAs were mainly associated with protein-coding genes. For example, flavonol synthase/flavanone 3-hydroxylase, isoflavone 2'-hydroxylase, polyphenoloxidase, disease resistance protein, E3 ubiquitin-protein ligase, basic proline-rich protein, aspartyl-tRNA synthetase, anthocyanidin 5,3-O-glucosyl-transferase, abscisic insensitive 1B, chitinase-like protein and cysteine-rich receptor-like protein kinase.

Detection of the expression of target genes using RT-PCR

MiRNAs would decrease the mRNA or protein level of their regulating target genes [1]. To explore whether the potential predicted miRNA targets could be regulated by miRNAs, the expression profiles of 10 target genes for one conserved miRNA and 5 novel miRNAs were investigated. As illustrated in Figure 3 and Figure 4, the predicted target genes *Morus015493* and *Morus018032* of miR156 were lowly expressed in male flower and highly expressed in leaf, which was opposite to the expression pattern of miR156. In addition, as shown in Figure 4 and Table S1, the predicted target genes *Morus012124*, *Morus012122*, *Morus012121* of miRn51 also had the opposite expression pattern with miRn51. Four novel miRNA-target gene pairs (*Morus008520* for miRn247, *Morus019289* for miRn67, *Morus011908* for miRn62, *Morus002508* and *Morus014466* for miRn157) also possessed the opposite expression pattern with each other.

**Discussion**

MiRNAs are important components in regulating plant physiological processes [1]. In the past several years, abundant conserved miRNAs and species-specific miRNAs were identified by high-throughput sequencing because of its high-throughput capacity in the detection of large-scale miRNAs and high sensitivity in the detection of minimally expressed miRNAs. In this study, sequencing of the three mulberry small RNA libraries was performed using Illumina technology. After analyzing millions of small RNA reads from these RNA libraries, 85 conserved mulberry miRNAs belonging to 31 families and 262 novel miRNAs at 371 loci were identified. After comparative analysis, several characteristics of conserved mulberry miRNAs were analyzed. A relationship between the degree of evolutionary conservation and the level of expression was observed in conserved miRNAs. As in previous studies, the majority of the conserved mulberry miRNA families identified here were evolutionarily conserved across plant species with high levels of expression. The less-conserved miRNA families (miR482, miR529, miR58, miR4376, miR1414, miR4995, and miR5529) showed lower abundance than the well-conserved miRNA families. The well- and less-conserved miRNA families may have evolved to play different roles in biological processes. The well-conserved mulberry miR164, miR167, miR156, miR172, miR159, miR166, miR171, miR172, and miR319 targeted NACs, ARFs, SPLs, APETALAs, MYBs, HD-ZIPIII,
SCLs, AP2s, and TCPs, respectively. These transcription factors are very important to plant growth and development. For example, in *A. thaliana*, miR164 and miR167 affect lateral root development and adventitious rooting, respectively [37,38]. miR167, miR159, miR160, and miR166 regulate the development of floral organs [39–46]. However, the targets of less-conserved miRNAs were mainly functional genes. It has recently been reported that miR482 and miR4376 target the NBS-LRR disease resistance gene and ACA10, respectively, and that they played a role in disease resistance and reproductive growth [47,48]. Although well- and less-conserved miRNA families played different roles, both were found to be very important. Specifically, they cooperated to regulate the biological processes in plants.

Many minimally expressed and species-specific miRNA have been discovered in the plant kingdom using high-throughput sequencing. This indicated that each plant has its own specific miRNAs, which may play specific roles in physiological processes. In the present study, the characteristics of pre-miRNA including stem-loop structures and MFE, served as identification criteria for novel mulberry miRNAs. A total of 90 mulberry miRNA* and 262 novel mulberry miRNAs were identified. miRNA* is the product of dicer-like 1 (DCL1) and partial complementary to miRNA [49]. It was once considered to be degraded shortly after production and to have no roles in biological processes [50]. Recent studies have shown that miRNA* have important functions related to physiologically relevant levels, even though they are expressed at much lower levels than their miRNAs [51,52]. It is speculated that the 90 low abundance miRNA* identified here might play important roles in various biological processes in mulberry plants. The novel mulberry miRNAs exhibited features different from those of conserved mulberry miRNAs. The expression levels of most novel mulberry miRNAs were very low, and they mainly targeted functional proteins. The predicted potential targets of mulberry novel miRNAs were involved in cellular processes, metabolic processes, response to stimulus, and metabolism. Research into possible targets in other plants may provide important clues to facilitate understanding of the function of these novel miRNAs. In the present study, one target of mno-miRn62 may be the disease resistance protein. This suggests that this novel miRNA may play a role in disease resistance in mulberry plants. In mulberries, flavanone 3-hydroxylase (F3H) has been found to participate in the anthocyanin biosynthesis pathway. The possible
targets of four novel miRNAs (mno-miRn14, mno-miRn137, mno-miRn176, and mno-miRn252) encoded F3H and may play a role in regulating anthocyanin biosynthesis. Further study into novel mulberry miRNAs may shed light upon their roles in mulberry biological processes. This may fill in the blanks with respect to current knowledge of biological processes involving conserved miRNAs. Novel miRNAs that work with the conserved miRNAs might regulate plant development and response to the environment more broadly and accurately than either set of miRNA alone.

Conclusions

This is the first comprehensive identification of conserved and novel miRNA in mulberry. The differential expression of miRNAs and the prediction of their target genes provide a basis for further understanding of mulberry miRNAs and the biological processes in which they are involved.

Supporting Information

Figure S1 Length distribution (A) and tissue distribution (B) of 85 conserved mulberry miRNAs. Numbers in B indicate the number of conserved miRNAs.

Table S1 The information of 262 novel mulberry miRNAs identified in three tissues.

Table S2 Tissue-biased conserved miRNAs and their target genes.

Table S3 Tissue-biased novel miRNAs and their target genes.

Table S4 Tissue-specific novel mulberry miRNA.

Table S5 Target genes of conserved mulberry miRNAs.

Table S6 Target genes of novel mulberry miRNAs.

Table S7 Primers used for stem-loop quantitative RT-PCR.

Table S8 The primers of target genes used for quantitative RT-PCR.

Author Contributions

Conceived and designed the experiments: LJ NJH. Performed the experiments: LJ DYZ BM. Analyzed the data: LJ XWQ. Contributed reagents/materials/analysis tools: LJ ZHX. Wrote the paper: LJ NJH. Designed the perl script used in analysis: XWQ.
References

1. Chen X (2009) Small RNAs and their roles in plant development. Annu Rev Cell Dev Biol 25: 21–44.
2. Ashkerman MJ, Sakai H (2003) Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. Plant Cell 15: 2730–2741.
3. Palatnik JF, Allen E, Wu X, Schommer C, Schwab R, et al. (2003) Control of leaf morphogenesis by microRNAs. Nature 425: 257–263.
4. Sunkar R, Li YF, Jagadeseeswaran G (2012) Functions of microRNAs in plant stress responses. Trends Plant Sci 17: 196–203.
5. Wu G, Poethig RS (2006) Temporal regulation of shoot development in Arabidopsis thaliana by miR136 and its target SPL3. Development 133: 3339–3347.
6. Chen X (2004) A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. Science 303: 2022–2025.
7. Wang JW, Wang LJ, Mao YB, Cai WJ, Xue HW, et al. (2005) Control of root cap formation by microRNA-targeted auxin response factors in Arabidopsis. Plant Cell 17: 2204–2216.
8. Lee RC, Feinbaum RL, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75: 835–843.
9. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001) Identification of novel genes coding for small expressed RNAs. Science 294: 853–858.
10. Lau NC, Lim LP, Weinstein EG, Bartel DP (2001) An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science 294: 838–862.
11. Grad Y, Aach J, Hayes GD, Reinhart BJ, Church GM, et al. (2003) Computational and Experimental Identification of C. elegans microRNAs. Mol Cell 11: 1235–1236.
12. Lai EC, Tomancak P, Williams RW, Kubin GM (2003) Computational identification of Drosophila microRNA genes. Genome Biol 4: R4.
13. Lee RG, Ambros V (2001) An extensive class of small RNAs in Caenorhabditis elegans. Science 294: 862–864.
14. Lim LP, Gslamer ME, Yekta S, Burge CB, Bartel DP (2003) Vertebrate microRNA genes. Science 299: 1596–1598.
15. Wang XJ, Reyes JL, Chua N-H, Gaareterd T (2004) Prediction and regulation of signaling of Arabidopsis thaliana microRNAs and their mRNA targets. Genome Biol 5: R65.
16. Lu C, Tej SS, Luo S, Handenenschuld CD, Meyers BC, et al. (2005) Elucidation of the small RNA component of the transcriptome. Science 309: 1567–1569.
17. Fahlgren N, Howell MD, Kasschau KD, Chapman EJ, Sullivan CM, et al. (2007) High-throughput sequencing, characterization and detection of new and conserved cucumber microRNAs. PLoS One. 6(5):e19523.
18. He N, Zhang C, Qi X, Zhao S, Tao Y, et al. (2013) Draft genome sequence of the mulberry tree Morus notabilis. Nat Commun 4: 2445.
19. Libault M, Farmer A, Joshi T, Takahashi K, Langley RJ, et al. (2010) An integrated transcriptome atlas of the crop model Glycine max, and its use in comparative analyses in plants. Plant J 63: 86–99.
20. Martinez G, Forment J, Llave C, Pallas V, Gomez G (2011) High-throughput sequencing, characterization and detection of new and conserved cucumber microRNAs. PLoS One 6(3):e19523.
21. Schwab R, Palatnik JF, Riedel M, Schmid M, et al. (2005) Specific effects of microRNAs on the plant transcriptome. Dev Cell 8: 517–527.
22. Chi X, Yang Q, Chen X, Wang J, Pan L, et al. (2011) Identification and characterization of microRNAs from peanut (Arachis hypogaea L.) by high-throughput sequencing. PLoS One 6: e27530.
23. Xu L, Wang Y, Xu Y, Wang L, Zhai L, et al. (2013) Identification and characterization of novel and conserved microRNAs in radish (Raphanus sativus L.) using high-throughput sequencing. Plant Sci 201–202: 108–114.
24. Ge A, Shangguan L, Zhang X, Dong Q, Han J, et al. (2012) Deep sequencing discovery of novel and conserved microRNAs in strawberry (Fragaria x ananassa). Physiol Plant. 140: 387–396.
25. Morig RD, Aksay G, Doloshinia E, Ebbhardt HA, Magrini V, et al. (2008) Comparative analysis of the small RNA transcriptionomes of Pinus contorta and Oryza sativa. Genome Res 18: 571–584.
26. Bonnet E, Wuyts J, Rouzé P, Van de Peer Y (2004) Evidence that microRNA precursors, unlike other non-coding RNAs, have lower folding free energies than random sequences. Bioinformatics 20: 2911–2917.
27. Guo HS, Xie Q, Fri JF, Chua NH (2005) MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for Arabidopsis lateral root development. Plant Cell 17: 1376–1386.
28. Gutierrez L, Russell JD, Picárur DI, Schorschbach J, Picárur M, et al. (2009) Phenotypic plasticity of adventitious rooting in Arabidopsis is controlled by complex regulation of AUXIN RESPONSE FACTOR transcripts and microRNA abundance. Plant Cell 21: 3119–3132.
29. Achard P, Herr A, Baulcombe DC, Harberd NP (2004) Modulation of floral development by a gibberellin-regulated microRNA. Development 131: 3357–3365.
30. Jung JH, Park CM (2007) MiR166/163 genes exhibit dynamic expression patterns in regulating shoot apical meristem and floral development in Arabidopsis. Planta 225: 1327–1338.
31. Kim J, Jung JH, Reyes JL, Kim YS, Kim SY, et al. (2005) microRNA - directed cleavage of ATTHB15 mRNA regulates vascular development in Arabidopsis inflorescence stems. Plant J 42: 84–94.
32. Liu X, Huang J, Wang Y, Khamma K, Xie Z, et al. (2010) The role of floral organs in carpels, an Arabidopsis loss-of-function mutation in MicroRNA160a, in organogenesis and the mechanism regulating its expression. Plant J 62: 416–429.
33. Mallory AC, Bartel DP, Bartel B (2005) MicroRNA-directed regulation of Arabidopsis AUXIN RESPONSE FACTOR1 is essential for proper development and modulates expression of early auxin response genes. Plant Cell 17: 1360–1375.
34. Nagpal P, Ellis CM, Weber H, Plowece SE, Barkawi LS, et al. (2005) Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. Development 132: 4107–4118.
35. Williams L, Grigg SP, Xie M, Christensen S, Fletcher JC (2005) Regulation of Arabidopsisshoot apical meristem and lateral organ formation by microRNA miR166g and its ARF-HD-ZIP target genes. Development 132: 3657–3668.
36. Wu MF, Tian Q, Reed JW (2006) Arabidopsis microRNA167 controls patterns of ARF6 and ARF8 expression, and regulates both female and male reproduction. Development 133: 4211–4218.
37. Shagrupas PV, Chen H-M, Patel K, Bond DM, Santos BA, et al. (2012) A microRNA superfamily regulates nucleotide binding site-leucine-rich-repeat rich and other mRNAs. Plant Cell 24: 1859–1874.
38. Yang W, Iwaya A, Zhang X, Wu Y, Zhang J, et al. (2011) Function and evolution of a microRNA that regulates a Ca2+–ATPase and triggers the formation of phase small injured RNAs in tomato reproductive growth. Plant Cell 23: 3125–2033.
39. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281–297.
40. Voigt O (2009) Origin, biogenesis, and activity of plant microRNAs. Cell 136: 669–678.
41. Okamura K, Phillips MD, Tyler DM, Duan H, Chou YT, et al. (2000) The regulatory activity of microRNAÁ species has substantial influence on microRNA and 3′ UTR evolution. Nat Struct Mol Biol 15: 534–536.
42. Zhang X, Zhao H, Gao S, Wang WC, Katiyar-Agarwal S, et al. (2011) Arabidopsis Argonaute 2 Regulates Inmate Immunity via miRNAs339-Mediated Silencing of a Gesh-Localized SNARE Gene, MEMB12. Mol Cell 42: 356–360.