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Microarray identification of conserved microRNAs in *Pinellia pedatisecta*

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ABSTRACT

Large numbers of microRNAs (miRNAs) reportedly play important roles in plant development. However, none has been reported in *Pinellia pedatisecta*, an important aroid medicinal plant that possesses the only pedate leaf blades and the largest tubers and inflorescences among all *Pinellia* species. To detect the miRNAs from *P. pedatisecta*, an *in situ* synthesized custom miRNA microarray was employed, following the verification for the presence of the miRNAs through reverse transcription polymerase chain reaction (RT-PCR) and the quantitative RT-PCR (qRT-PCR). A total of 99 miRNAs belonging to 22 miRNA families were identified. The RT-PCR was applied to 14 miRNAs detected to validate the microarray results. The qRT-PCR that targeted seven miRNAs showed different expression levels of miRNAs in different tissues. The current research is the first to report on the miRNAs in *P. pedatisecta* and will enable further investigation of their roles in *P. pedatisecta* development.

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1. Introduction

Pinellia pedatisecta Schott is a monocotyledonous perennial herbaceous plant of the *Araceae* family that is widely used as an important herb in traditional Chinese medicine. *P. pedatisecta* is native to Asia and North America, and is the only species of *Pinellia* with pedate leaf blades divided into seven to eleven lanceolate leaflets and lacks a transverse septum inside its spathe. The tubers of *P. pedatisecta*, widely used as medicine for the treatment of lymph node enlargement, urinary tract infection, nausea, and vomiting, among others, as well as its inflorescences, are the largest among all *Pinellia* species.

MicroRNA (miRNA) plays important regulatory roles in plants by targeting mRNA (Bartel, 2004; Zhang et al., 2006a). Most of the targeted mRNAs are key components of the complex network of regulatory pathways in plant physiologic activities, including cell differentiation, apical meristem maintenance, leaf polarity establishment, morphogenesis, floral organ identity determination, and so on (Molnár et al., 2007; Mallory et al., 2004a; Baker et al., 2005; Mallory et al., 2004b; Chen, 2004; Juarez et al., 2004). Thousands of plant miRNAs have been documented in the Sanger miRNA database (http://www.mirbase.org/) and the plant miRNA database (http://bioinformatics.cau.edu.cn/PMRD/). However, no miRNA has been reported in *P. pedatisecta*, whose genomic information is unknown. The identification of miRNA from *P. pedatisecta* would be very helpful for further research on the molecular mechanisms of its physiologic characteristics and its role in the development and protection of *P. pedatisecta*.

Numerous miRNAs have been identified from many plants such as *Arabidopsis thaliana*, *Fragaria ananassa*, *Solanum tuberosum*, *Oryza sativa*, *Glycine max*, *Sorghum bicolor*, *Nicotiana tabacum*, and *Zea mays*, among others (Mica et al., 2006; Zeng et al., 2010; Li et al., 2009; Yang et al., 2010; Sunkar et al., 2005; Liu et al., 2008; Dezulian et al., 2005; Yin et al., 2008). Although plants are divided into either monocotyledonous or eudicotyledonous clades, many plant miRNAs remain conserved between these two ancient plant groups (Zhang et al., 2006b; Axtell and Bartel, 2005). The typical eudicotyledonous and monocotyledonous plants are represented by 30 miRNA families containing 88 miRNAs that are highly conserved in *A. thaliana* and *O. sativa* (Li et al., 2007). The majority of the members of the 42 miRNA families in *A. thaliana* can also be found in *O. sativa*, *Z. mays*, and *S. bicolor* (Griffiths-Jones, 2004). As a family of highly conserved small non-coding RNAs, many miRNAs are reportedly conserved in the homologous sequences from all lineages of land plants including bryophytes, lycopods, ferns, and seed plants (Floyd and Bowman, 2004; Sunkar and Jagadeeswaran, 2008). These include miR156/157, miR160, miR159, miR319, miR165/166, miR390, and miR408, which are found in seed plants and in primitive land plants, such as *Physcometrella* and *Selaginella* (Axtell and Bartel, 2005; Floyd and Bowman, 2004; Arazi et al., 2005; Axtell et al., 2007). This finding allows the application of experimental strategies for miRNA identification in plants with unknown genomes based on the conserved miRNA from well-researched plant species. As a high-throughput, precise, and efficient technology, microarray has been widely applied in the identification of conserved miRNA in plants with sparse genomic information (Li et al., 2009; Zhang et al., 2008).

Abbreviations: RT-PCR, reverse transcription polymerase chain reaction; qRT-PCR, Quantitative realtime polymerase chain reaction; PM, perfect match; MM, one nucleotide mismatch.

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In the current study, a μParaflo® microfluidic array (LC Sciences, USA) containing 1957 sequence-unique probes that correspond to the miRNA sequences reported in the Sanger miRBase Release 16 was used to detect the conserved miRNA from *P. pedatisecta*. The microarray hybridization result was verified using reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR). The results of the current study could help elucidate the roles of miRNAs in diverse biological processes, and could serve as guides for further in-depth transgenic research on *Pinellia*.

2. Results

To validate the reliability of the miRNA microarray, various specific controls were used in the experiment. Two groups of quality-control probes, distributed in 16 representative hybridization areas to monitor the microarray, were employed. The hybridization results reveal that the signals of the nucleotide probes, a nucleotide mismatch (MM) with the synthetic spiked-in RNA (20mer), were clearly reduced to background levels, as represented by the bar graphs shown in Fig. 1. Those of the probes perfect matched (PM) with the synthetic spiked-in RNA (20mer) were relatively higher (Fig. 1). All signals from the quality-control probes distributed in the different hybridization areas on the microchip shown in Fig. 1A are consistent with those in Fig. 1B. Furthermore, the 5S rRNA signals used as the internal controls were also strong and stable. These findings indicate that the miRNA microarray system is highly sensitive and specific.

Up to 99 sequence-unique miRNAs belonging to 22 miRNA families (miR156, miR159, miR164, miR165, miR166, miR167, miR168, miR171, miR319, miR396, miR397, miR399, miR528, miR529, miR535, miR894, miR1432, miR1450, miR156l, miR2919, miR2936, and miR3946) were detected from the leaf tissue of *P. pedatisecta* using miRNA microarray, which contains 1957 sequence-unique probes complementary to the known mature miRNAs from 43 plant species (Supplement 1). The microarray data have been submitted to Gene Expression Omnibus (Accession No. GSE33915).

Based on the analysis of the hybridization data in the current study, the detectable rates of the identified miRNAs from the monocotyledonous *Sugarcane officinarum*, *Hordeum vulgare*, *S. bicolor*, *Festuca arundinacea*, and *Brachypodium distachyon* were 60.00%, 50.00%, 43.28%, 35.71%, and 27.78%, respectively, whereas those from *Z. mays*, *O. sativa*, and *Triticum aestivum* were only 16.34%, 14.20%, and 7.69%, respectively. For the dicotyledonous *Brassica napus*, *Ricinus communis*, *Brassica rapa*, *Aquilegia coerulea*, and *Populus trichocarpa*, the detectable rates were up to 46.15%, 41.67%, 40.00%, 38.24%, and 35.96%, respectively. Some miRNAs from gymnosperms, bryophyte, and ferns were also detected in *P. pedatisecta*.

Among the miRNAs detected in *P. pedatisecta*, 15 miRNA families (miR159, miR167, miR156, miR164, miR166, miR168, miR397, miR171, miR535, miR399, miR528, miR396, miR1432, miR156l, and miR2919) are conserved in *O. sativa*, and 11 miRNA families (miR159, miR167, miR156, miR164, miR166, miR168, miR319, miR397, miR171, miR399, and miR1450) were detected in *P. trichocarpa*. The sequences of aly-miR159a, aly-miR164a, and aly-miR167a are deeply conserved in 13, 11, and 10 plant species, respectively.

To verify the result of the miRNA microarray in the present study, RT-PCR was conducted to detect the presence of miRNA in the *P. pedatisecta* leaf tissues using stem-loop primers targeted to 14 specific miRNAs (aly-miR164a, aly-miR165a, aly-miR168a, aly-miR319a, aqc-miR167, ath-miR2936, bdi-miR171c, csi-miR3946, osa-miR1432, osa-miR156l, osa-miR166e, osa-miR399a, ppt-miR529e, and ptc-miR1450) identified by microarray analysis (Supplement 2). The RT-PCR results with negative controls can be found in Supplement 3. The results of the amplified miRNA fragments are consistent with the predicted length (Fig. 2). The sequencing results of all cloned fragments show that the amplicons were complementary or reverse complementary to the corresponding miRNA sequences (Supplement 1), including the primers, which also reflected that the stem-loop primers are specific and suitable for miRNA research in *P. pedatisecta*.

Simultaneously, the designed primers specific to the seven miRNAs (ptc-miR159f, ptc-miR397b, ppt-miR894, aqc-miR535, aly-miR156g, osa-miR528, and gma-miR396e) were used for qRT-PCR analysis (Supplement 2). The results of the qRT-PCR show that the identified miRNAs are present in the different tissues of *P. pedatisecta*.

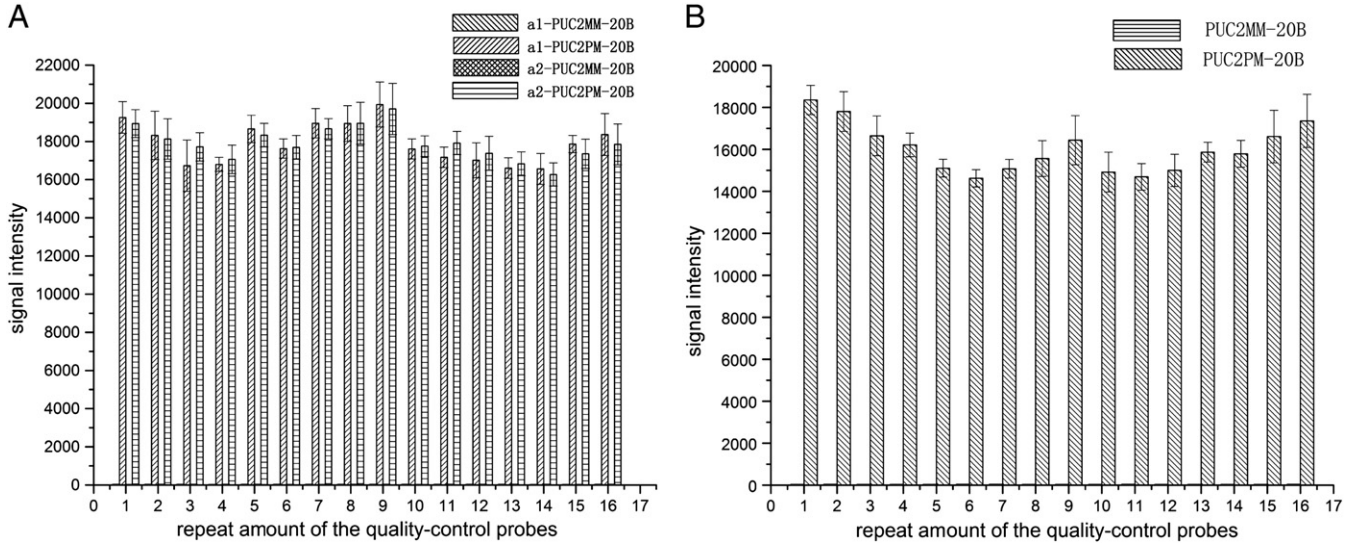

Fig. 1. The hybridization signals of the two groups of quality-control probes in the microarray. A) The probes a1-PUC2PM-20B and a2-PUC2PM-20B are perfectly matched with the spiked-in RNA presented significantly stronger signals, whereas the signals of the mismatch probes a1-PUC2MM-20B and a2-PUC2MM-20B were obviously reduced to background levels, which are barely noticeable in the figure. B) The results from the second group of quality-control probes PUC2PM-20B and PUC2MM-20B were consistent with those in the former group presented in panel A. All probes were distributed in 16 hybridization areas. The error bars indicate the standard deviations obtained from the pixels of the digital Image.

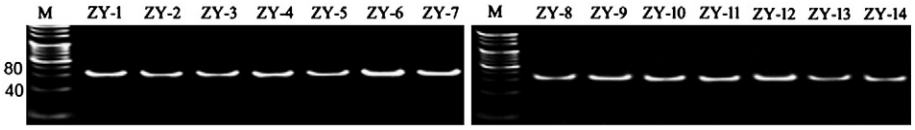

Fig. 2. The resulting PCR amplicons of the miRNAs cloned in 8% PAGE. M, DNA ladder marker; ZY-1, aly-miR164a; ZY-2, aly-miR 165a; ZY-3, aly-miR168a; ZY-4, aly-miR319a; ZY-5, aqc-miR167; ZY-6, ath-miR2936; ZY-7, bdi-miR171c; ZY-8, csi-miR3946; ZY-9, osa-miR 1432; ZY-10, osa-miR156l; ZY-11, osa-miR166e; ZY-12, osa-miR399a; ZY-13, ppt-miR529e; ZY-14, ptc-miR1450.

The miRNAs ptc-miR159f, ptc-miR397b, osa-miR528, and gma-miR396e presented similar expression levels in the leaves and stalks, with the highest expression levels presented in the leaf tissue. The expression level of ptc-miR397b in the leaf tissue is nearly eightfold that in the stalks. By contrast, the aqc-miR535 presented the highest expression level in the stalk tissue. However, no remarkable difference was found in the ppt-miR894 and aly-miR156g in the leaves and stalks (Fig. 3).

3. Discussion

MiRNA plays versatile and important roles in post-transcriptional regulation by inhibiting gene translation or degrading coding mRNAs. In plants, most target mRNAs only contain one single miRNA-complementary site, and most corresponding miRNAs perfectly complement these sites and cleave the target mRNAs. Several experimental and genetic analyses have indicated that miRNAs are essential for plant development, and response to hormone signaling and environmental stress (Gou et al., 2011; Sunkar, 2010). Previous studies have provided evidence that many miRNAs are evolutionarily conserved in the vegetable kingdom. The analysis of the data obtained on the miRNA data base revealed that 8 miRNA families (miRNA159, miRNA167, miRNA156, miRNA164, miRNA166, miRNA168, miRNA319, and miRNA397) are highly conserved in more than 10 of the 43 plant species selected. In particular, miRNA159, miRNA167, and miRNA156 can be found in 23, 21, and 20 plants, respectively. These data are consistent with those reported previously (Zhang et al., 2006b; Axtell and Bartel, 2005), confirming the evolutionary conservation of plant miRNAs.

The evolutional conservation of miRNAs suggests that they play important and conserved functions in plant development (Palatnik et al., 2003; Ding et al., 2010). For instance, miRNA166 downregulates the mRNA accumulation of *REVOLUTA*, *PHABULOSA*, and *PHAVOLUTA* to determine the abaxial fate in *Arabidopsis*. Furthermore, miRNA166 regulates the mRNA accumulation of *ROLLED LEAF* 1 to determine the adaxial or abaxial polarity of the developing leaves of *Z. mays*. In *O. sativa*, the less-expressed miRNA166 could cause pleiotropic phenotypes including narrow, rolled, and outward-folded leaves in *OsDCL1IR* transformants, consistent with the conserved function of miRNA166 in *Z. mays* and *Arabidopsis* (Juarez et al., 2004; Kim et al., 2005; Emery et al., 2003; Mallory et al., 2004c; Liu et al., 2005; Kidner and Martienssen, 2004). In the current study, the miRNA166 family detected in *P. pedatisecta* was highly conserved in the 14 plant species selected including *Arabidopsis lyrata*, *O. sativa*, and *Z. mays*. These results indicated that miRNA166 possesses similar regulatory functions in *P. pedatisecta*.

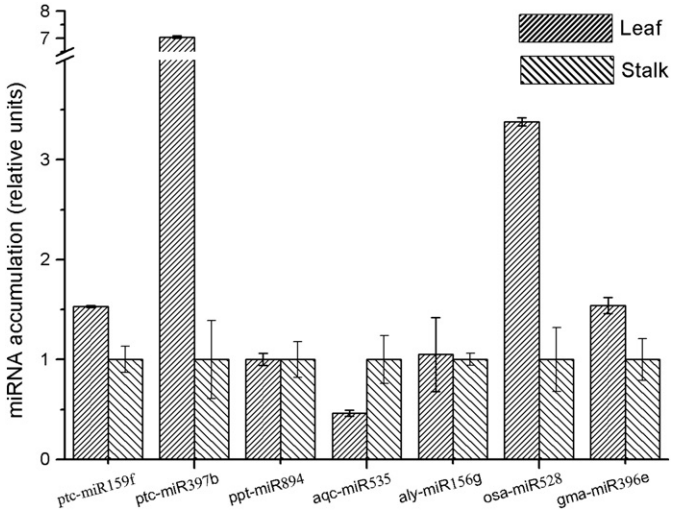

Fig. 3. The expression level of each miRNA in the stalks was set to 1, whereas those in the leaves was quantified relatively using the $2-\Delta\Delta Ct$ method. The error bars indicate the standard deviations obtained from the independent experiments.

MiRNA397, a highly conserved miRNA, is predicted to target laccases, which are copper-containing oxidase enzymes found in many plants; the target sites are conserved in *Arabidopsis*, rice, *N. tabacum*, *Populus*, and other flowering plants (Luo et al., 2006; Sunkar and Zhu, 2004; Shen et al., 2011). Furthermore, it is reportedly detectable in the leaves, roots, and young seedlings, but not in the stems and inflorescence tissues (Sunkar and Zhu, 2004). However, similar to miRNA167, miRNA169, miRNA170, miRNA171, miRNA172, miRNA319, miRNA396, and miRNA398, miRNA397 is highly accumulated in the leaves (Gustafson et al., 2005). These data are concordant with the result in the current study (Fig. 3), which suggests the similar regulatory functions of miRNA397 in *P. pedatisecta*, such as response to oxidative stress, drought stress, cold stress, nutrient-deprivation, and copper homeostasis (Sunkar, 2010; Shen et al., 2011).

MiRNA156, present in 21 plants including *P. pedatisecta*, has been predicted to target the mRNAs of *SPL* transcription factors to regulate the reproductive and floral growth. The transformants of overexpressed miRNA156 presented smaller plants, late flowering, a tendency to lose apical dominance, and initiation of more leaves with shorter plastochrons compared with the wild-type *Arabidopsis* (Schwarz et al., 2008). MiRNA319, is highly conserved in 14 plant species selected in this experiment and plays an important role in maintaining leaf phenotype by regulating the members of *TCP* transcription factors in *Arabidopsis* (Palatnik et al., 2007). MiRNA167 regulates plant development by downregulating the proteins that bind to the auxin-responsive cis elements (Ru et al., 2006). MiRNA164 regulates the NAC-domain targets required for the proper formation and separation of adjacent embryonic, vegetative, and floral organs (Mallory et al., 2004a; Sieber et al., 2007). These conserved miRNAs are very important to cell fate determination in plants (Rhoades et al., 2002), which could be very helpful in the cultivation and research of *P. pedatisecta*, and the development and protection of *Pinellia*, a genus widely used as an important herb in traditional Chinese medicine (Wong and Sagar, 2010).

In plants, miRNA-guided gene regulation plays a key role in diverse biological processes, including various aspects of plant development and adaptation to biotic and abiotic stresses; thus, their identification in different plant species is essential to the understanding of post-transcriptional gene regulation (Sunkar, 2010; Mallory and Vaucheret, 2006; Jones-Rhoades et al., 2006). In the current study, 99 sequence-unique miRNAs belonging to 22 microRNA families (Supplement 1), which are highly conserved in monocotyledonous, dicotyledonous, gymnosperm, and algal species, were identified. Osa-miRNA528, a

novel miRNA found in *O. sativa*, whose target is still unknown (Liu et al., 2005), exists in relatively high expression levels in leaf tissues of *P. pedatisecta*. The detection of miRNAs in *P. pedatisecta* could facilitate the investigation their regulatory roles in plant physiologic processes. The roles of these conserved miRNAs in *P. pedatisecta* need to be tested through overexpression or silencing in transgenic plants.

In conclusion, miRNAs from *P. pedatisecta* were identified and were found to play important roles in plant development. The detected miRNAs will enable further in-depth testing of their roles in *P. pedatisecta* and in transgenic research on *Pinellia*.

4. Materials and methods

4.1. Plant materials and reagents

The *P. pedatisecta* plants were grown in a greenhouse at Zhejiang Sci-Tech University. The mirVana™ miRNA kit was purchased from Ambion, USA. The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The platinum SYBR Green qPCR SuperMix-UDG was purchased from Invitrogen.

4.2. RNA extraction and microarray assay

The total RNA was extracted from the leaf tissues of *P. pedatisecta* using the mirVana™ miRNA Kit, which was then size-fractionated using a YM-100 microcon centrifugal filter (Millipore). The small RNAs (<300 nt) collected were 3′-extended with a poly(A) tail using poly(A) polymerase, and then ligated with an oligonucleotide tag for the subsequent fluorescent dye staining using tag-specific staining Af5 dyes.

The miRNA microarray (LC Sciences) design was based on 2521 well-characterized miRNAs from *Aegilops taushii*, *A. coerulea*, *Arachis hypogaea*, *A. lyrata*, *A. thaliana*, *Bombax ceiba*, *B. distachyon*, *B. napus*, *Brassica oleracea*, *B. rapa*, *Carica papaya*, *Citrus reticulata*, *Chlamydomonas reinhardtii*, *Citrus clementina*, *Citrus sinensis*, *Citrus trifoliata*, *F. arundinacea*, *Gossypium herbaceum*, *Gossypium hirsutum*, *G. max*, *Gossypium raimondii*, *Glycine soja*, *H. vulgare*, *Lotus japonicus*, *Malus domestica*, *Medicago truncatula*, *O. sativa*, *Picea abies*, *Populus euphratica*, *Physcomitrella patens*, *Pinus taeda*, *P. trichocarpa*, *Phaseolus vulgaris*, *Ricinus communis*, *S. bicolor*, *Solanum lycopersicum*, *S. officinarum*, *Selaginella moellendorffii*, *T. aestivum*, *Triticum dicoccum*, *Vigna unguiculata*, *Vitis vinifera*, and *Z. mays*. These cover monocotyledonous, dicotyledonous, algal, and gymnosperm species, including the 1957 sequence-unique mature miRNAs, were employed on a μParaflo™ microfluidic microchip using *in situ* parallel synthesis and RNA hybridization-optimized probes. Each miRNA probe was repeated twice on the microarray. Eighteen 5S rRNAs that served as internal reference were chosen from *A. thaliana*, *C. reticulata*, and *O. sativa* with an equal amount. Eight quality-control probes were synthesized to perfect match or one nucleotide mismatch with eight external spiked-in synthetic RNA (20mer).

4.3. MicroRNA microarray experiment and data analysis

According to the protocol provided by LC Sciences, overnight hybridization was performed at 34 °C using the μParaflo microfluidic chip station (LC Sciences), and then washed in 0.1× saline-sodium phosphate-ethylenediamine tetraacetate buffer. After spin-drying, the slides were scanned using a fluorescence image scanner (GenePix 4000B, Axon/Molecular Device).

The hybridization image was digitized using the Array-Pro image analysis software (Media Cybernetics). The data were analyzed by subtracting the background, and the signals were normalized based on the LOWESS program (Yang et al., 2002). The sign values met the following criteria: higher than the background value plus a threefold standard deviation and a coefficient of variation less than 0.5 were considered valuable and reliable.

4.4. Reverse transcription polymerase chain reaction (RT-PCR)

To verify the data obtained from the microarray, RT-PCR was performed by employing a stem-loop primer designed based on those described by Chen et al. (2005). After the total RNA extracted from the *P. pedatisecta* leaves and DEPC H_2O mixture was heated to 70 °C for 10 min and quenched on ice for 5 min, the stem-loop primer and the remaining reagents were added, incubated at 16 °C for 30 min, followed by 60 cycles of 20 °C for 30 s, 42 °C for 30 s, and 50 °C for 1 s, then 75 °C for 15 min, and finally maintained at 4 °C. PCR was performed using the reverse transcription products as templates. All reactions, including the negative controls (no template), were performed in duplicate. The PCR-amplified products were separated by electrophoresis through 2.5% agarose gel and visualized by ethidium bromide staining.

The PCR fragments were recovered from the agarose gel and ligated into the pEasy™-T1 cloning vector (Beijing TransGen Biotech Co., Ltd.) for transformation into *Escherichia coli* DH5α competent cells. After the plasmids were isolated from the selected colonies, they were confirmed by PCR and sequenced by Sangon Biotech (Shanghai).

4.5. Quantitative real-time RT-PCR (qRT-PCR)

After the total RNA was extracted from the leaf and stalk tissues of three *P. pedatisecta* samples under the same conditions, they were exposed to RNase-free DNase I. The cDNA of the miRNAs was then synthesized by RTase M-MLV (TaKaRa) using stem-loop primers through the aforementioned protocol. The qRT-PCR reactions containing SYBR Green Mix (Invitrogen) were performed at 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s on an Applied Biosystem 7900. The reactions of each sample, including the negative controls, were conducted in triplicate. The relative amount of each miRNA to 5.8S rRNA (GenBank accession no. AF469037.1) was determined using the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = (\text{Nominal } C_t \text{ Mean})_{\text{Tissue L}} - (\text{Nominal } C_t \text{ Mean})_{\text{Tissue S}}$; Nominal C_t = "Sample C_t Median" − "Control C_t Median" + "Median Control C_t". Median Control C_t is defined as the median value of "Control C_t Median" for all samples. Tissue S presents the expression level of each miRNA in the stalks, whereas the expression levels in the leaves were quantified relative to it. The standard deviations of the data were obtained from nine independent experiments.

Supplementary materials related to this article can be found online at doi:10.1016/j.gene.2012.01.075

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