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ORIGINAL ARTICLE

Duplication and expression analysis of multicopy miRNA gene family members in *Arabidopsis* and rice

Danhua Jiang[1, 2], Changsong Yin[1], Aiping Yu[1, 3], Xiaofan Zhou[1], Wanqi Liang[1, 4], Zheng Yuan[1], Yun Xu[1], Qingbo Yu[1], Tieqiao Wen[2], Dabing Zhang[1, 4]

[1]SJTU-SIBS-PSU Joint Center for Life Sciences, School of Life Science and Biotechnology, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China; [2]School of Life Science, Shanghai University, 99 Shangda Road, Shanghai 200444, China; [3]College of Life and Environment Sciences, Shanghai Normal University, 100 Guilin Road, Shanghai 200234, China; [4]Key Laboratory of Agricultural Genetics and Breeding, Agro-biotech Research Center, Shanghai Academy of Agricultural Sciences, 2901 Beidi Road, Shanghai 201106, China

To understand the expansion of multicopy microRNA (miRNA) families in plants, we localized the reported miRNA genes from *Arabidopsis* and rice to their chromosomes, respectively, and observed that 37% of 117 miRNA genes from *Arabidopsis* and 35% of 173 miRNA genes from rice were segmental duplications in the genome. In order to characterize whether the expression diversification has occurred among plant multicopy miRNA family members, we designed PCR primers targeting 48 predicted miRNA precursors from 10 families in *Arabidopsis* and rice. Results from RT-PCR data suggest that the transcribed precursors of members within the same miRNA family were present at different expression levels. In addition, although miR160 and miR162 sequences were conserved in *Arabidopsis* and rice, we found that the expression patterns of these genes differed between the two species. These data suggested that expression diversification has occurred in multicopy miRNA families, increasing our understanding of the expression regulation of miRNAs in plants.

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Introduction

MicroRNA (miRNA) comprises an extensive family of important regulatory noncoding RNAs of approximately 21-22 nucleotides in length and is found both in animals and in plants [1-6]. These are generated by RNase III-like enzymes from long primary transcripts encoded in intergenic regions (IGRs) or introns of the mRNA primary transcripts [5,7-9]. Mature miRNAs can downregulate gene expression by pairing with messages of protein-coding genes to promote mRNA cleavage, or repression of productive translation upon incorporation into a ribonucleoprotein complex (miRNP) similar to the RNA-induced silencing complex (RISC) [10-14]. Indeed, the vast majority of the predicted or verified miRNA targets encode members of large families of transcription factors that regulate development [15]. Mutants that lack miRNA pathway proteins, such as DICER-LIKE 1 (DCL1), ARGONAUTE 1 (AGO1) and HUA ENHANCER 1 (HEN1), exhibit morphological defects in *Arabidopsis* and rice, suggesting an important role for miRNA in plant development [8,14,16-20]. Recently, more and more plant miRNAs have been reported for their specialized developmental functions, such as miR159, miR-JAW, miR160, miR162, miR165/166, miR168, and miR172. These miRNAs are involved in flower anther development [21], rosette leaf curvature [22], organ polarity [23, 24], floral organ identity and flowering time [25, 26], auxin response [27], and feedback regulation of the miRNA

Correspondence: Dabing Zhang
Tel: +86-21-34205073; Fax: +86-21-34204869;
E-mail: zhangdb@sjtu.edu.cn
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pathway [19, 28].

In *Arabidopsis* and rice, exceeding 100 miRNAs have been cloned or predicted. Although some have a single copy in the genome, many miRNA sequences correspond to multiple loci of 2-19 copies (http://microrna.sanger.ac.uk/cgi-bin/sequences/browse.pl). The mature miRNA sequence in each locus is predicted to pair with a nearby genomic segment, forming a dsRNA stem-loop structure, which is contained in miRNA precursors (pri-miRNA and pre-miRNA) and is required for Dicer processing. The mature miRNA can then be processed from either the 5' or the 3' arm of this structure [9]. This type of multiple loci phenomenon has been widely observed in plants and animals [9, 29-31]. Although recent research has demonstrated that nearly all the miRNA genes of *Arabidopsis* are expressed [32], little is known, in plants, about whether the expression or function is different among members within the same miRNA family.

In this paper, we observed that the expansion of miRNAs to multiple loci might be partly resulted from genome duplication in *Arabidopsis* and rice, and RT-PCR was utilized to estimate the expression of miRNA members within gene families. We demonstrated there were different expression levels of the precursors of multiple members, within the same family. In *Arabidopsis* and rice, differences in the expression patterns of the conserved miRNA families in two species suggest that the regulation of miRNA expression in plants is highly complex. Our data help to further elucidate the expression regulatory mechanisms of miRNA genes in plants.

Materials and methods

Plant materials and growth condition

Wild-type *Arabidopsis thaliana* plants (Columbia) and transgenic plants were grown in long days (16 h light, 8 h dark) at 22 °C with cool white light. Rice (*Oryza sativa*, japonica cultivar 9522) was grown in a greenhouse at 30 °C during the day and 24 °C at night.

miRNA genes location and duplication analysis

Duplicated block map of *Arabidopsis*, provided by TIGR (http://www.tigr.org/tdb/e2k1/ath1/arabGenomeDups.html), and rice predicted chromosome duplicated regions deduced from bioinformatics analysis of transcription factors [33] were used for analyzing the reported miRNA gene location of *Arabidopsis* and rice (http://microrna.sanger.ac.uk/cgi-bin/sequences/browse.pl) to deduce segmental duplications on the chromosomes. In these maps (Figure 1C and D), the boxes of the same color indicate the duplicated regions.

The stem-loop regions of miRNA genes retained as duplicated pairs or nonduplicated pairs were selected for alignment using the Clustal X program (http://www-igbmc.u-strasbg.fr/BioInfo) [34]. GendDoc (http://www.psc.edu/biomed/genedoc) [35] was used to highlight the conserved and similar nucleotide sequences (Figure 2).

Primer design

The hairpin structures of each miRNA were referenced from a miRNA database (http://microrna.sanger.ac.uk/cgi-bin/sequences/browse.pl). PCR primers were designed with Primer Express version 2.0 software (Applied Biosystems, Foster City, CA, USA). RT-PCR primers for detecting precursors were designed to target the nucleotide sequence within the predicted hairpin structure of these miRNA family members, so that the designed primer pairs could amplify both the long primary transcripts (pri-miRNA) and the short processed transcripts (pre-miRNA) (Figure 3). The term "miRNA precursors" refers to both pri-miRNA and pre-miRNA [36], and the criteria used to design sense and antisense primers were similar to what has been reported previously [36].

The designed antisense primers were used as gene-specific primers to promote the RT reaction. To ensure accurate amplification of each miRNA precursor, at least one primer sequence of the primer pair was designed to partly mismatch the mature miRNA or its complementary (miRNA*) sequence (Figure 3). The amplicon size of miRNA precursors ranged from 63 to 179 bp based on the various lengths of the predicted miRNA hairpin structures in plants. To minimize differences in RT efficiency, we utilized the same gene-specific primers between different members from the same family as possible. In the case of At-miR159, At-miR162, At-miR165/166, Os-miR162, Os-miR166, and Os-miR172 families, the same antisense primer (complementary to mature miRNA sequence) was designed for each member of the same family. Since the mature miRNA

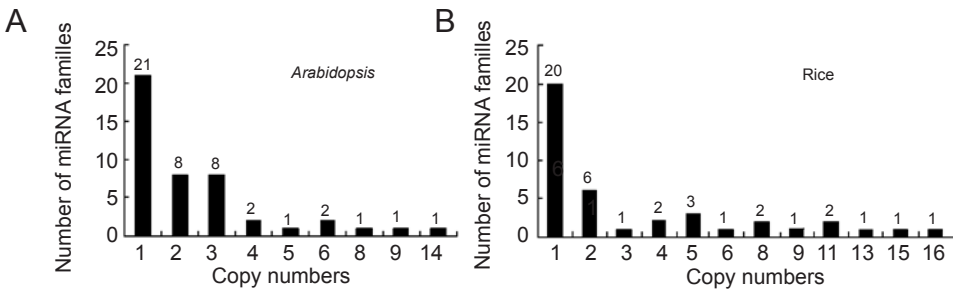
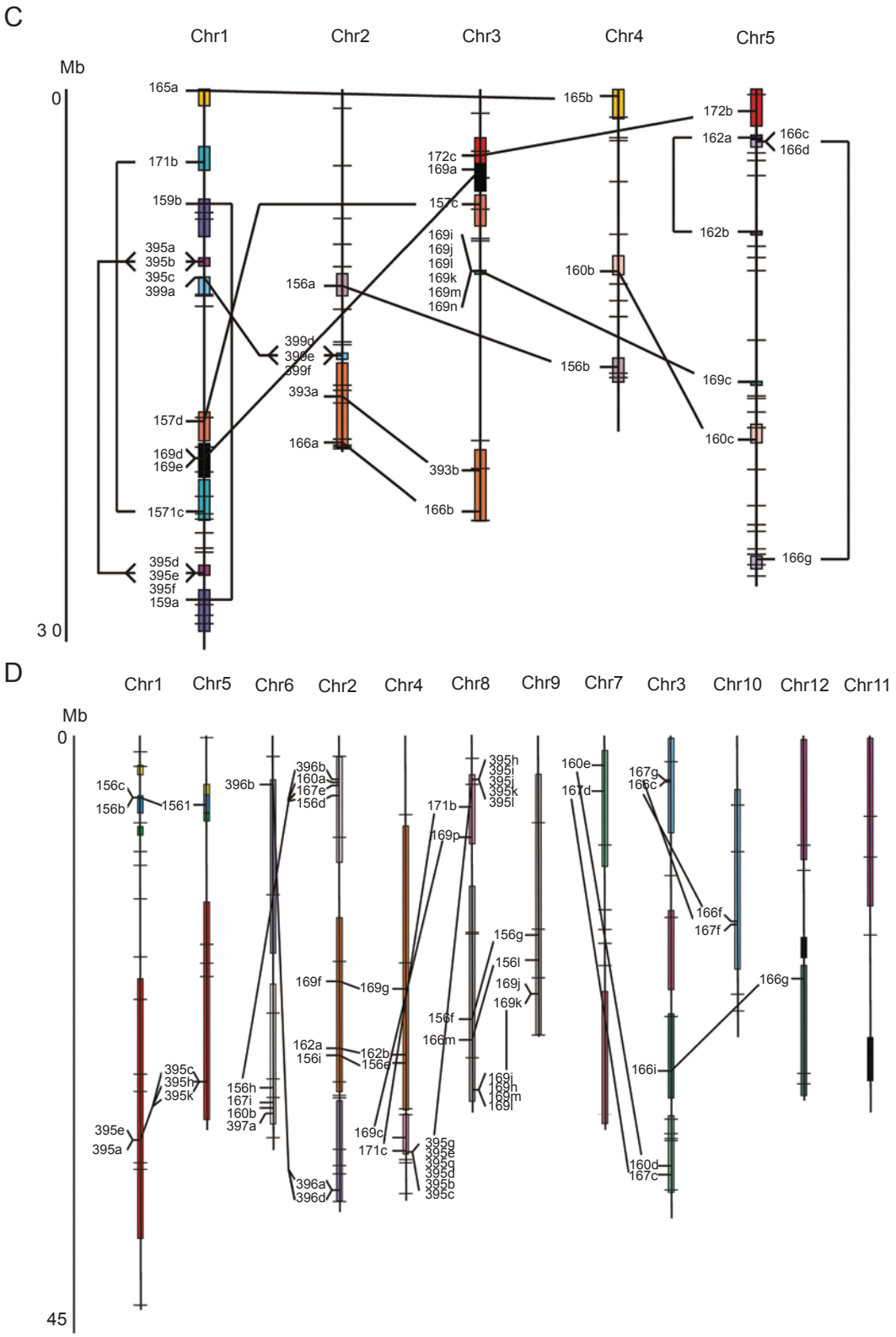


Figure 1 miRNA gene location and duplication analysis in *Arabidopsis* and rice genomes. **(A)** Statistical chart of the copy number of the identified miRNAs in *Arabidopsis* genome. **(B)** Statistical chart of the copy number of the identified miRNAs in rice genome.



(C) and **(D)** Chromosome locations and duplication events for miRNAs indicated in *Arabidopsis* and rice genomes. Segmentally duplicated regions in the chromosomes are indicated by boxes of the same color. The scale of *Arabidopsis* **(C)** is adapted from the scale available on the NCBI database, and that of rice **(D)** is from TIGR, both are in megabases (Mb). Connecting lines mark the specific cases in which there is a strong correlation between duplicated genomic regions and the presence of miRNA genes in the same family.

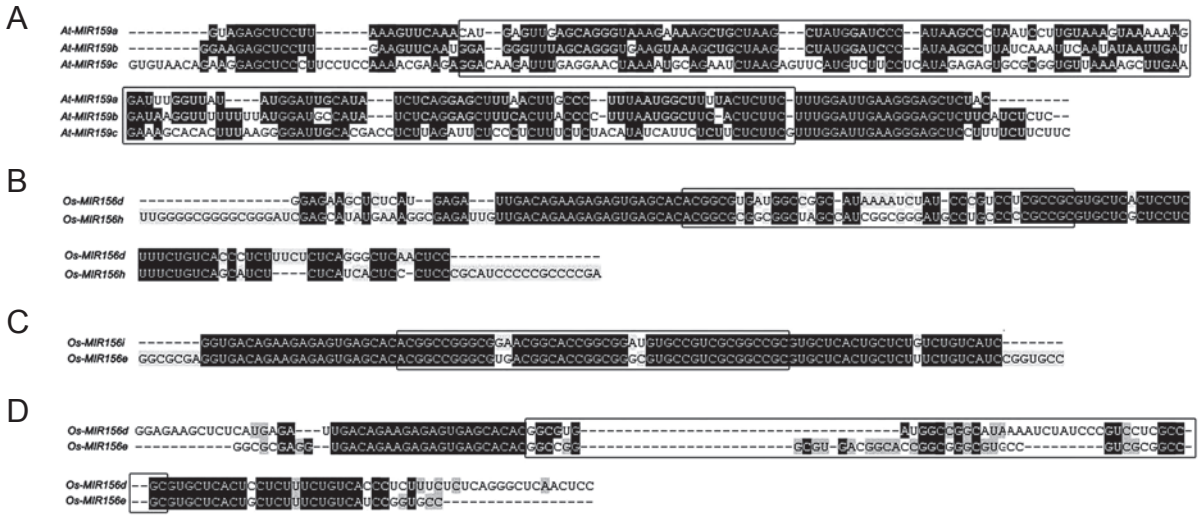


Figure 2 Alignment of the predicted pre-miRNAs sequences within deduced duplicated pairs. The loop sequence of the pre-miRNA was in the box. **(A)** Alignment of *At-MIR159a* (chromosome 1: 184 bp from 27716894 to 27717077), *At-MIR159b* (chromosome 1: 196 bp from 6220639 to 6220834), and *At-MIR159c* (chromosome 2: 225 bp from 19001705 to 19001929), the *At-MIR159a* and *At-MIR159b* were predicted to be the duplicated pair. **(B)** and **(C)** Sequence alignment of duplicated pairs from Os-miR156 family. **(B)** *Os-MIR156d* (chromosome 2: 129 bp from 4512881 to 4513009) and *Os-MIR156h* (chromosome 6: 101 bp from 26510033 to 26510133). **(C)** *Os-MIR156i* (chromosome 2: 90 bp from 24065107 to 24065196) and *Os-MIR156e* (chromosome 4: 104 bp from 24650629 to 24650732). **(D)** Alignment of sequences from different duplicated pairs of Os-miR156 family, *Os-MIR156d* and *Os-MIR156e*.

sequence was located on the 5' arm of the hairpin structure of the members of At-miR157, At-miR160, At-miR168, and Os-miR160 families, and the complementary (miRNA*) sequence on the 3' arm was partly variable among those members, making it was difficult to design one antisense primer for reverse transcription. For this reason, different antisense primers (At-MIR157aRTA, At-MIR157bRTA, At-MIR157cRTA, At-MIR157dRTA, At-MIR160aRTA, At-MIR160bRTA, At-MIR160cRTA, At-MIR168aRTA, At-MIR168bRTA, Os-MIR160a/b/c/dRTA, Os-MIR160eRTA, and Os-MIR160fRTA) were designed for each member respectively. The difference between the annealing temperatures of these antisense primers was within 2 °C and the GC content difference was less than 10%. In order to analyze *At-MIR162a* and *At-MIR162b* precursors, one sense primer At-MIR162RTS was designed on the At-miR162* location (sequence complementary to At-miR162). Since the At-miR162* sequence had high similarity between the *At-MIR162a* and *At-MIR162b*, the sense primer At-MIR162RTS and antisense primer At-MIR162RTA could be used to monitor both precursors of *At-MIR162a* and *At-MIR162a*. In order to specifically detect the *At-MIR162a* precursor, another sense primer, At-MIR162aRTS, that mismatched the At-miR162* sequence, was designed on the *At-MIR162a* stem-loop region; thus the primer pair At-MIR162aRTS and At-MIR162RTA could be used specifically for amplifying the *At-MIR162a* precursor. The Tm value of all primer pairs ranged from 54 to 59 ºC, and all primers were confirmed to be capable of amplifying the target DNA fragments by using genomic DNA as a template.

RT-PCR

Total cellular RNA was isolated by guanidine thiocyanate/phenol-chloroform extraction as described [37], and treated with DNaseI (Promega, Shanghai, China). The RNA concentration was quantified using absorbance at 260 nm. First strand cDNA synthesis was performed with 3 μg RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's recommendations with either oligod$T_{(18)}$ primer or the gene-specific primers.

RT reaction was heated (70 °C for 5 min) to denature the RNA, incubated (37 °C for 5 min) to anneal the primers, and then cooled on ice. The remaining reagents (5 × reaction buffer, dNTPs, RNase inhibitor, M-MuLV reverse transcriptase) were then added and mixture was incubated (42 °C for 60 min). The reaction was heated (80 °C for 10 min) to inactivate reverse transcriptase and the RT products were purified by phenol/chloroform (1:1) and resolved in 100 μl of water.

PCR was performed using sense (RTS) and antisense (RTA) primers of each miRNA precursor. Reaction volumes (30 μl) contained 1×PCR Buffer, 200 μM of dATP, dGTP, dCTP, and dTTP (TaKaRa, Dalian, China), 1 mM of each primer, 2.5 μl diluted RT template in water, and 2.5 units of Taq DNA polymerase (TaKaRa). Amplifications were performed utilizing a PTC-100 thermocycler (MJ Research, Waltham, MA, USA) as follows: one step of 5 min at 94 ºC, 34 cycles of 94 °C for 30 s, 54 – 58 °C (based on the Tm values of the primers) for 30 s, and 72 °C for 30 s. PCR amplicons were resolved in either 2.2% agarose or 10% polyacrylamide electrophoresis in 0.5 × TBE buffer and stained with ethidium bromide. Gel bands were evaluated by direct observation on a UV transilluminator, and then recorded using a computer imaging system. Each PCR pattern was verified in triplicate; negative controls (no template and no reverse

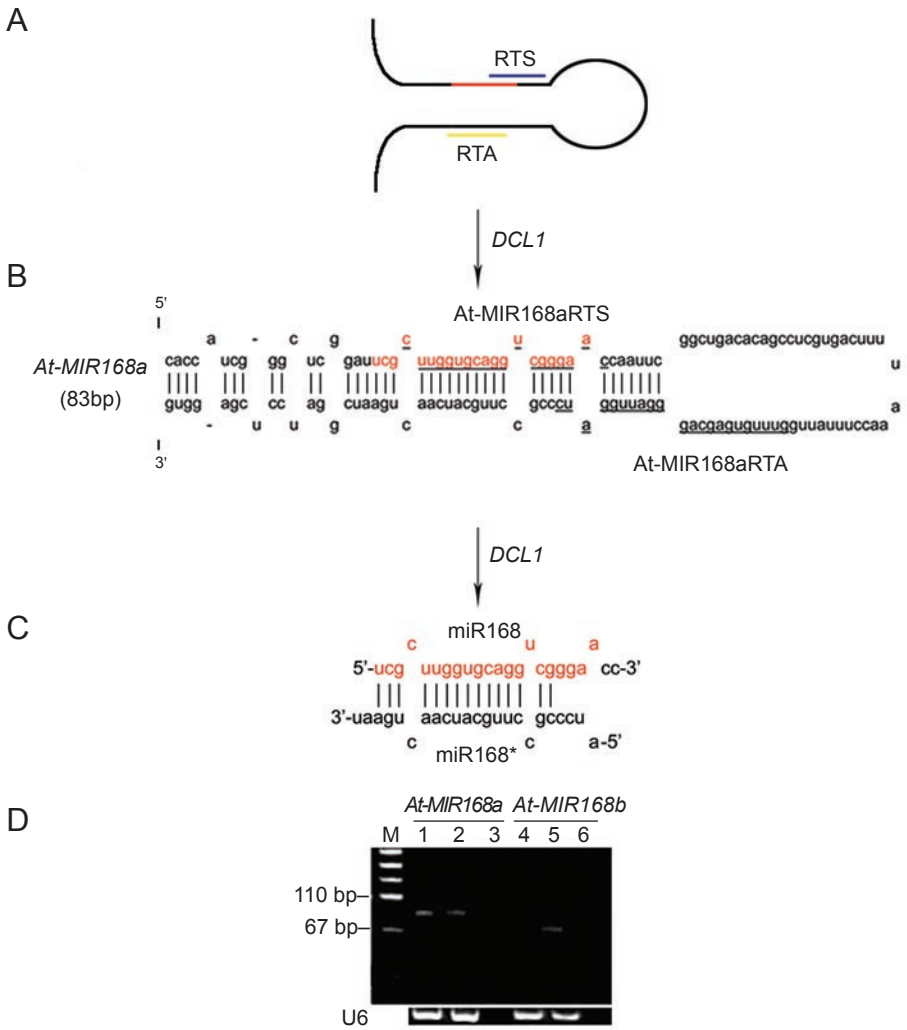


Figure 3 Primer design and miRNA processing in plants taking *At-MIR168a* as an example. **(A)** Plant miRNA primary transcripts (pri-miRNA) are proposed to be stepwise processed by the same enzyme *DCL1* in nucleus that generated **(B)** pre-miRNA and **(C)** miRNA:miRNA* duplex, respectively. Both the pri-miRNA and pre-miRNA contain the stem-loop structure. The mature miRNA sequence is in red, and the hybridization sequence of sense and antisense primers are underlined. **(D)** RT-PCR detection of At-miR168 family, the PCR products for cDNA (lanes 1, 4), genomic DNA (lanes 2, 5), and the same reactions without RT (lanes 3, 6) were resolved on a 10% PAGE gel.

transcription) were also included for each gene. The PCR products amplified with cDNA or genomic DNA were subcloned into pMD18-T vector (TaKaRa) and were confirmed by sequencing.

5′ RACE PCR and computational identification of conserved motifs in promoter region

5' RACE PCR was performed with 5' Full RACE Core set (TaKaRa) following the manufacturer's instructions (Figure 4A). 5' RACE PCR primers were also designed according to the manufacturer's instructions. RT primers were located less than 60 bp downstream of the 3' end of the predicted stem-loop sequence (Figure 4C and 4D) and were phosphorylated at the 5' end, facilitating cDNA self-ligation. Based on the manufacturer's suggestion, the length of RT primers was designed to be less than 20 nt, with Tm values of approximately 45 °C. First round PCR primers were located in the predicted stem-loop structure of each member, and second round PCR primers were located with a maximal extension of 40 bp over the 5' or 3' end of the predicted stem-loop structure. The range of Tm values between the nested PCR primer pairs was within 4 ºC.

Total RNA was extracted as described above; cDNA was generated with 3 mg RNA using gene-specific primers, e.g. At-165aRTP, At-165bRTP, At-168aRTP, At-168bRTP, Os-162aRTP, and Os-162bRTP, and self-ligated by T4 RNA ligase. The first round PCR amplification using first PCR primer pairs was performed with RT products, respectively. To obtain the specific products, the nested PCR was performed using the first round PCR dilution with the second PCR primer pairs. Amplified products were gel purified and cloned into pMD18-T vector for sequencing.

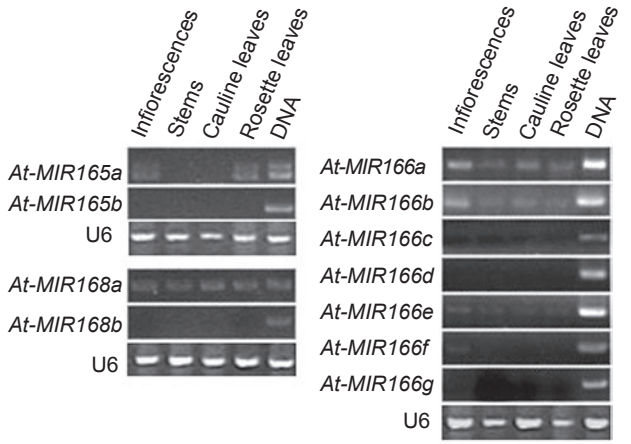


Figure 4 Expression of some miRNA family members tested in *Arabidopsis* inflorescences, stems, cauline leaves, and rosette leaves. Three micrograms total RNA from inflorescences, stems, cauline leaves, and rosette leaves was analyzed by RT-PCR. *At-MIR166a*, *At-MIR166b*, *At-MIR166e,* and *At-MIR168a* could be detectable in all the tested organs, whereas the amplicon of *At-MIR165a* and *At-MIR166f* could only be visible in some organs. Other tested miRNA variants could not be detected in all the organs.

Conserved sequences in the promoter regions were identified using a Bioprospector program as described previously [32].

Transgenic vector and plant transformation

To test whether the mature miR168 could be generated from both predicted stem-loop structures of *At-MIR168a* and *At-MIR168b*, a Col-0 genomic DNA fragment with 276 bp containing the predicted *At-MIR168a* hairpin precursor was cloned using the sense primer MIR168aS (5' ttt tgg tac cCC CAT TAC AAA ACC TAG AAC 3' from 162619 to 162638bp, Genbank Accession. AL161550) 81 bp upstream of the 5' end of the hairpin structure and the antisense primer MIR168aA (5' ttt gaa ttc TAA AGA AGG AGA AGC GTA GA 3', from 162894 to 162875 bp, Genbank Accession. A L161550) 57 bp downstream of the 3' end of the hairpin structure, containing the *KpnI* and *EcoRI* restriction sites, respectively. A 305 bp genomic DNA fragment of *At-MIR168b* with 81 bp upstream from the 5' end of hairpin structure and 100 bp downstream from the 3' end of hairpin precursor was amplified with genomic DNA from Col-0 plants using the sense primer MIR168bS (5' ttt ggt acc TGC CGG ATT TGG TAA CTG TTT TCT CC 3' from 40450 to 40425 bp, Genbank Accession. AB020744) and the antisense primer MIR168bA (5' ttt gaa ttc CAA TAC CGA ATC AAT CAA ATC ACA CAG 3' from 40146 to 40172 bp, Genbank Accession. AB020744), respectively; *kpnI* and *EcoRI* restriction enzyme sites were also introduced into this genomic DNA fragment.

The two PCR products were gel purified and then subcloned into the pMD18-T vector for sequencing. The resulting clones were digested with *Kpn* Ⅰ and *EcoR* Ⅰ. Recovered DNA fragments were ligated into a pMON530 Vector (Monsanto, St. Louis, MO, USA) digested with the same set of enzymes. The fragments were inserted between the cauliflower mosaic virus (CaMV) *35S* promoter and the *nos* terminator.

Wild-type *Arabidopsis* plants were transformed with *Agrobacterium* strain GV3101 carrying the constructed plasmid utilizing the flower-dipping method [38].

Northern blot analysis of mature miR168

Total RNAs were isolated from rosettes of wild-type and transgenic plants. Total RNA (10 μg) was resolved in a 19% polyacrylamide gel and were blotted to a Nylon membrane (Schleicher&Schuell, Dassel, Germany). DNA oligonucleotides (5' TTC CCG ACC TGC ACC AAG CGA 3', from 21 to 1 bp, Genbank Accession. AJ493652) complementary to mature miR168 were end-labeled with [α-^{32}p] ATP and T_4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) to generate high-specific activity probes. Hybridization was carried out as described [29] and blots were rehybridized with a designed probe (5' GAG GGA TGC AAC ACG AGG ACT T 3', from 296 to 317 bp, Genbank Accession. AY130753) complementary to 5S rRNA.

Results

Chromosome location and duplication analysis of multi-copy miRNA genes in Arabidopsis and rice genomes

On the basis of location information provided by the Sanger Institution (http://microrna.sanger.ac.uk/cgi-bin/sequences/browse.pl), we realized that approximately 50% of the identified miRNAs corresponded to multiple loci in *Arabidopsis* (24/45) (Figure 1A) and in rice (21/41) (Figure 1B) genome. According to this location information, we localized a total of 117 deduced miRNA genes from *Arabidopsis* and 173 identified miRNA genes from rice on five *Arabidopsis* chromosomes and 12 rice chromosomes, respectively. Furthermore, we investigated the relationship between the designated genome-wide intra and interchromosomally duplicated areas and the location of miRNA genes belonging to the same family. Our data suggest that many miRNA genes were located within the proposed genomic segmental duplicated regions (Figure 1C and 1D). Among the 173 miRNA genes in rice, 65% (113/173) of the miRNA genes resided on the deduced duplicated genomic segments [33], whereas 35% (60/173) were retained as duplicated pairs (Figure 1D). Similarly, the ratios in *Arabidopsis* were 82% (96/117) (http://www.tigr.org/tdb/e2k1/ath1/arabGenomeDups.html) and 37% (43/117) (Figure 1C). In addition, we observed clusters of related miRNA genes (one cluster of *MIR169* and two clusters of *MIR395*) in both *Arabidopsis* and rice genomes, which may indicate the occurrence of tandem gene duplication events (Figure 1C and 1D).

Moreover, the similarity of the miRNA family genes distributed in the duplicated pairs was observed by aligning the predicted stem-loop sequence of these miRNA genes. For example, alignment of the *At-MIR159* family (Figure 2A) revealed that the nucleotide sequence similarity was

Table 1 RT-PCR detection of tested miRNA family members in *Arabidopsis* and rice. The detectable precursors were in grey shade

Arabidopsis		Rice	
miRNA genes	RT-PCR detection	miRNA genes	RT-PCR detection
At-MIR157a	U		
At-MIR157b	U	nh	
At-MIR157c	D		
At-MIR157d	D		
At-MIR159a	D	*Os-MIR159a*	
At-MIR159b	D	*Os-MIR159b*	nt
At-MIR159c	U	*Os-MIR159c*	
		Os-MIR159d	
		Os-MIR159e	
		Os-MIR159f	
At-MIR160a	D	*Os-MIR160a*	D
At-MIR160b	D	*Os-MIR160b*	U
At-MIR160c	D	*Os-MIR160c*	D
		Os-MIR160d	D
		Os-MIR160e	U
		Os-MIR160f	U
At-MIR162a	U	*Os-MIR162a*	D
At-MIR162b	D	*Os-MIR162b*	D
At-MIR165a	D	*Os-MIR166a*	D
At-MIR165b	U	*Os-MIR166b*	D
At-MIR166a	D	*Os-MIR166c*	U
At-MIR166b	D	*Os-MIR166d*	D
At-MIR166c	U	*Os-MIR166e*	U
At-MIR166d	U	*Os-MIR166f*	U
At-MIR166e	D	*Os-MIR166g*	D
At-MIR166f	U	*Os-MIR166h*	D
At-MIR166g	U	*Os-MIR166i*	U
		Os-MIR166j	U
		Os-MIR166k	D
		Os-MIR166l	U
		Os-MIR166m	D
At-MIR168a	D	*Os-MIR168a*	nt
At-MIR168b	U	*Os-MIR168b*	
At-MIR172a		*Os-MIR172a*	D
At-MIR172b	nt	*Os-MIR172b*	U
At-MIR172c		*Os-MIR172c*	D
At-MIR172d		*Os-MIR172d*	D
At-MIR172e			

D: detectable, U: undetectable, nh: no homology, nt: not tested

high (76.1%) between *At-MIR159a* and *At-MIR159b*, both of which had previously been proposed to be involved in a duplicated event. Whereas, less similarity (49.3%) was observed for those not proposed to be a duplicated pair, such as *At-MIR159a* and *At-MIR159c*. Similar results were observed in rice *Os-MIR156* family members (Figure 2B and 2C), in which the sequence similarity between the stem-loop sequences of the same duplicated pairs was above 70% (81.7% between *Os-MIR156i* and *Os-MIR156e* and 72.9% between *Os-MIR156d* and *Os-MIR156*l*h*), but less than 40% similarity was noted between the sequences from different duplicated pairs (Figure 2D). Even though the loop region sequences of miRNA genes were proposed to be divergent for undergoing no functional constraint [40], we also observed the conservation among members of a duplicated pair (Figure 2A-2C). These data serve to validate the duplication events, especially when considering that the loop region is usually less conserved in miRNA genes.

Monitoring the expression of miRNA family members with multicopy

Through above results, it was observed that different evolution might occur among those miRNA genes in the same family. Therefore, we proposed that the expression or functional diversification of miRNA family members with multicopy existed. To prove this point, we chose to detect the expression level of miRNA precursors transcribed from each miRNA gene. Firstly, a sensitive detection system, RT-PCR, was set up and employed to amplify and detect miRNA precursors in plants (Figure 3). The PCR sensitivity was tested and proved to be sufficient for detecting at least 100 copies of the target fragment using genome DNA as template (data not shown). RT reactions were carried out with either Oligod$T_{(18)}$ primer or gene-specific primers, respectively. We found that only three transcribed miRNA precursors, e.g. *At-MIR166b*, *Os-MIR162a,* and *Os-MIR162b,* could be amplified with the use of Oligod$T_{(18)}$ primed RT products (data not shown), whereas a greater number of miRNA precursors could be amplified by employing gene-specific primers for the RT reaction (Table 1). This result may be explained by the fact that many miRNA primary transcripts in plants could have been processed rapidly and that the 3' end structures of the transcribed products disappeared quickly [41]. All the expected RT-PCR products were confirmed by sequencing. These results indicated that the established PCR system can be used for monitoring miRNA precursors in plants.

Since there are difficulties in designing primer pairs of the hairpin structures to discriminate the miRNA family members, finally 6 miRNA families with 23 predicted members from *Arabidopsis* and 4 miRNA families with 25 predicted members from rice were selected for detect-

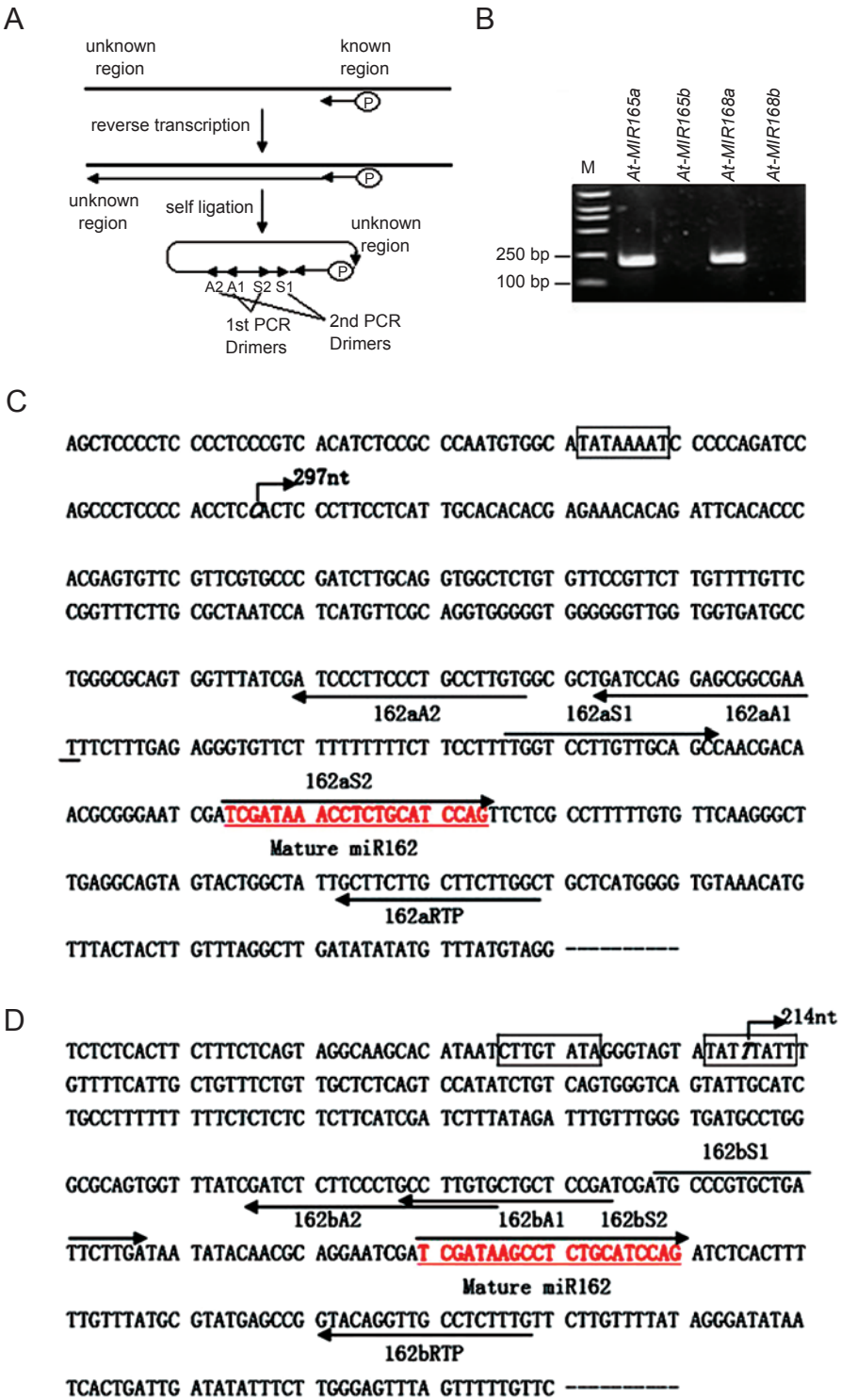


Figure 5 Scheme of 5' RACE PCR analysis. **(A)** Illustration of 5' RACE PCR method used in this study. RT-PCR products were self-ligated, followed by two rounds of PCR amplification. **(B)** Image of agarose gel showing the 5' RACE PCR products of *At-MIR165a* (216bp), *At-MIR165b*, *At-MIR168a* (206bp), and *At-MIR168b*. **(C)** and **(D)** Sequences around the transcription start sites of *Os-MIR162a* and *Os-MIR162b*. The primers used for reverse transcription and PCR are indicated. The filled arrow indicates the transcription start sites and distance from 5' end of mature miRNA. The sequence of mature miRNA is in red; TATA box-like sequence was identified upstream from the transcribed region.

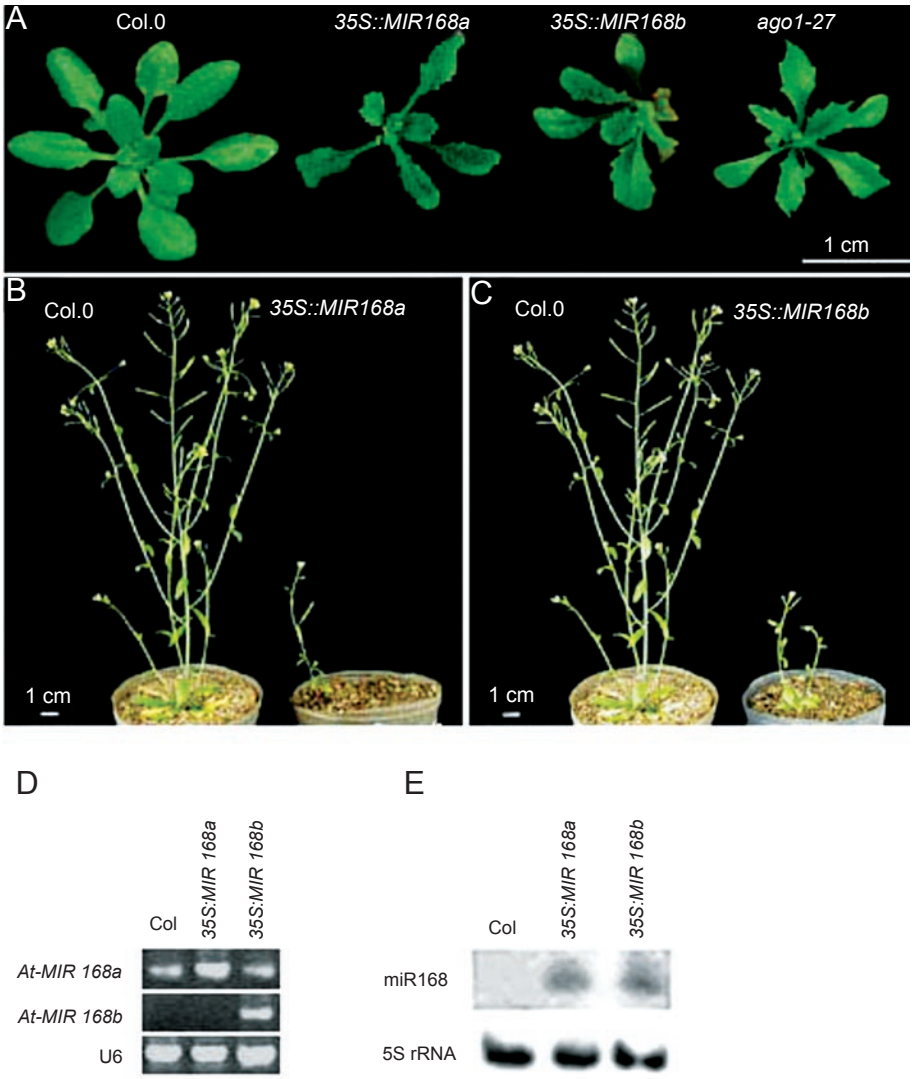


Figure 6 Development defects caused by overexpressing of *At-MIR168a* and *At-MIR168b*, and expression levels of miR168 precursors and mature miR168 in *35S::MIR168a* and *35S::MIR168b* transformants. **(A)-(C)** Similar *ago1* mutant phenotypes were observed in both *35S::MIR168a* and *35S::MIR168b* transformants. **(D)** MiR168 precursors accumulation detected using RT-PCR in transgenic plants. The *At-MIR168b* precursor could be detected in *35S::MIR168b* transformants. **(E)** Mature miR168 accumulation in both *35S::MIR168a* and *35S::MIR168b* transformants.

ing the expression level. These miRNAs were predicted to correspond to 2, 3 , 4, 6 , 7, and 13 copies in the genome. The results showed that the expression levels of miRNA members within the same family were different in plants (Table 1), which suggested that the expression diversification might occur among them. Interestingly, the precursors of *At-MIR159a* and *At-MIR159b*, both of which had been proposed to be a duplicated pair, could be detected, whereas, the precursor of *At-MIR159c* could not be detected, implying similar expression regulatory mechanisms may be performed on miRNA genes in the same duplication pair.

To further investigate the detailed expression pattern of miRNA family variants, At-miR165/166 family and At-miR168 family were selected to monitor the corresponding transcribed precursors in cauline leaves, rosette leaves, stem, and inflorescences (Figure 4). The miRNA precursors of *At-MIR166a*, *At-MIR166b*, *At-MIR166e,* and *At-MIR168a* could be monitored in all the tested organs, suggesting that these might play roles in all these organs. The amplicon of one miRNA precursor, *At-MIR166f*, which could not be detected in the sample of mixed tissues, was visible in inflorescences, suggesting that *At-MIR166f* might function specifically during reproductive stage. Addition-

ally, we interestingly observed that the tested miRNA variants were expressed highly in inflorescences (Figure 4), reflecting their possible important roles in reproductive organs.

To date, approximately 30 miRNA families have been found to be conserved in *Arabidopsis* and rice (http://microrna.sanger.ac.uk/cgi-bin/sequences/browse.pl). Interestingly, although the precursors of all the At-miR160 and Os-miR162 family members could be detected, some of their homologous members, e.g. *At-MIR162a, Os-MIR160b*, *Os-MIR160e,* and *Os-MIR160f,* were not detected in our experiment (Table 1), implying there might exist differences in miRNA expression regulation between monocot and dicot plants.

The 5'-terminal structure of miRNA primary transcripts in Arabidopsis and rice

In order to further confirm the RT-PCR results, we utilized a 5' RACE PCR method to monitor the expression of some miRNA genes including At-miR165 and At-miR168 family members. Our data demonstrate that only the specific amplified DNA fragments, corresponding to the transcripts of *At-MIR165a* and *At-MIR168a,* were obtained after the two rounds of PCR amplification, but no product was generated to the *At-MIR165b* and *At-MIR168b* transcripts (Figure 5B). This result indicated that the transcript levels of *At-MIR165a* and *At-MIR168a* were high enough to be detected, whereas the levels of *At-MIR165b* and *At-MIR168b* were not high enough after the two rounds of PCR amplification. These data were also consistent with the RT-PCR analysis results of the At-miR165 and At-miR168 families. The same method was performed on Os-miR162 family members and both members were detected, similar to RT-PCR result.

Based on the nucleotide sequence analysis of the 5' RACE PCR amplified products, we observed two additional G bases in the 5'-terminal of tested transcripts, which were proposed to be the G cap structure. Moreover, TATA box-like sequences were observed approximately 10-30 bp upstream from the transcription start sites of *Os-MIR162a* and *Os-MIR162b* (Figure 5C and 5D). The 5'-terminal characters of *Os-MIR162a* and *Os-MIR162b* were similar to those tested miRNA genes in *Arabidopsis* [32], demonstrating that both in monocots and in dicots, miRNA genes may be transcribed by RNA polymerases II [32, 42].

Overexpressed precursors of both At-MIR168a and At-MIR168b can result in higher accumulation of miR168

In the current study, we also investigated whether functional diversification occurred between miRNA members in the same family. Two genomic DNA fragments of approximately 300 bp containing the stem-loop structure of *At-MIR168a* and *At-MIR168b* were cloned under the control of the CaMV 35 s promoter. Both constructs were subsequently introduced into wild-type plants. As reported previously, miR168 can negatively regulate *AGO1* expression [19]; therefore, after selection with kanamycin, we observed that some transgenic plants displayed developmental defects similar to the *AGO1* mutants in both *35S::MIR168a* and *35S::MIR168b* transformants. Total RNA was extracted from rosette leaves of wild type, *ago1* mutant like *35S::MIR168a,* and *35S::MIR168b*, and the levels of miR168 were determined. Higher accumulation levels of precursors and mature miR168 were noted in *35S::MIR168a* and *35S::MIR168b* plants (Figure 6D and 6E). This suggested that even though the predicted precursor of *At-MIR168b* was beyond detection, the stem-loop structure of *At-MIR168b* still contain the sequence information responsible for mature miR168 generation. In addition, similar phenotypes caused by overexpressing both *At-MIR168a* and *At-MIR168b* also indicated that no obvious functional diversification occurred between *At-MIR168a* and *At-MIR168b* (Figure 6A-6C).

Discussion

The discovery of miRNAs in both plants and animals expands our knowledge of gene modulation. In plants, miRNA genes are mostly discrete, independent transcription units, and about 50% of miRNAs are predicted to be multiple loci in the genome, similar to that seen with protein-coding genes (recent rice sequencing data showed that 29% of protein-coding genes appear in gene families) [43]. The evidence here suggesting that the expansion of miRNA genes in plants resulted from genome segmental and tandem duplication events during evolution is similar to that of many protein-coding gene families, especially transcription factor families [33, 44]. Thus, these kinds of duplication events are proposed to have contributed to the expansion of both protein-coding gene families and miRNA gene families. The same expression status of *At-MIR159a* and *At-MIR159b* in a duplication pair increases this possibility too.

Recently, the transcripts of most miRNA genes in *Arabidopsis* have been detected by 5' RACE and 3' RACE approaches [32]. However, it is difficult to demonstrate expression differences of miRNA genes utilizing this method as multiple (2-4) rounds of PCR amplification are not able to estimate differences in the initial cDNA templates. Detection of miRNA precursors by gel-based methods is also difficult, given the rapid processing of miRNA precursors in plants. In the current study, we have developed a method to better detect expression differences of miRNA family members in plants utilizing RT-PCR approach, and

demonstrate that it can successfully identify different transcript levels within same miRNA family members. Even though our system could not detect very low level precursor molecule, these tested results provide direct evidence that the expression levels of miRNA members within the same family were different in plants, from which we infer that complex mechanisms may regulate the expression of these members.

The ability to detect some precursors may result from the fact that the corresponding loci may have been transcribed at relatively high levels in tested plant samples. On the other hand, undetectable precursors may be due to their low expression levels in tested samples or due to the fact that some loci may only be expressed in specific cells or during specific developmental stages, such as *At-MIR166f*, the transcript of which could only be detected in inflorescence. Additionally, some may be pseudogenes. Since the expression of nearly all miRNA genes in plants is proposed to be independently regulated by their own promoters, many miRNAs may have developmental stage-specific or cell (tissue, organ)-specific expression patterns determined by their promoter region. In animals, transcription studies of *let-7* in *C. elegans* demonstrated that the temporal regulatory element (TRE) located at about 1200 nt upstream from the mature *let-7* coding sequence was required for temporal upregulation of let-7 [45]. Recently, studies of the At-miR164 family showed that *At-MIR164a* and *At-MIR164b* participate in lateral root development [46], whereas *At-MIR164c* was demonstrated to control petal number in early flowers [47], indicating the nonredundant expression of the three members.

For protein-coding gene families, both the expression and functional diversification may occur among family members. Similar events may also occur with miRNA family members. However, here, molecular data have demonstrated that both the hairpin structures of *At-MIR168a* and *At-MIR168b* could contribute to mature miR168 processing. In addition, with phenotype analysis data, it was suggested that there is no obvious functional diversity between the *At-MIR168a* and *At-MIR168b*.

In order to gain further insight into the expression and functional diversity of multicopy miRNA families in plants, additional research is needed, like investigating the expression patterns of different miRNA members within the same families in details. Analysis of the miRNA promoter regions in plants may also reveal conserved elements required for regulating miRNA expression patterns.

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