

# Genetic framework for flowering-time regulation by ambient temperature-responsive miRNAs in *Arabidopsis*

Hanna Lee<sup>1</sup>, Seong Jeon Yoo<sup>1</sup>, Jeong Hwan Lee<sup>1</sup>, Wanhui Kim<sup>1</sup>, Seung Kwan Yoo<sup>1</sup>, Heather Fitzgerald<sup>2,3</sup>, James C. Carrington<sup>2,3</sup> and Ji Hoon Ahn<sup>1,\*</sup>

<sup>1</sup>Creative Research Initiatives, School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea, <sup>2</sup>Department of Botany and Plant Pathology and <sup>3</sup>Center for Genome Research and Biocomputing, Oregon State University, Corvallis, OR 97331, USA

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## ABSTRACT

Flowering is the primary trait affected by ambient temperature changes. Plant microRNAs (miRNAs) are small non-coding RNAs playing an important regulatory role in plant development. In this study, to elucidate the mechanism of flowering-time regulation by small RNAs, we identified six ambient temperature-responsive miRNAs (miR156, miR163, miR169, miR172, miR398 and miR399) in *Arabidopsis* via miRNA microarray and northern hybridization analyses. We also determined the expression profile of 120 unique miRNA loci in response to ambient temperature changes by miRNA northern hybridization analysis. The expression of the ambient temperature-responsive miRNAs and their target genes was largely anticorrelated at two different temperatures (16 and 23°C). Interestingly, a lesion in *short vegetative phase (SVP)*, a key regulator within the thermosensory pathway, caused alteration in the expression of miR172 and a subset of its target genes, providing a link between a thermosensory pathway gene and miR172. The miR172-overexpressing plants showed a temperature-independent early flowering phenotype, suggesting that modulation of miR172 expression leads to temperature insensitivity. Taken together, our results suggest a genetic framework for flowering-time regulation by ambient temperature-responsive miRNAs under non-stress temperature conditions.

## INTRODUCTION

Small RNAs including microRNAs (miRNAs) are a class of regulatory molecules that play an important role throughout the plant life cycle. MiRNAs are non-coding RNAs (20–24 nucleotides in length) that negatively regulate expression of their target genes via either sequence-specific degradation or translational repression (1). The important roles of plant miRNAs in plant growth and development have been demonstrated by the pleiotropic developmental abnormalities seen in many miRNA biogenesis mutants. One of the most severe cases is the *dcl1* mutant (2), in which miRNA production is strongly inhibited. The *dcl1* mutants are embryo-lethal, implying that miRNA production and subsequent negative regulation by miRNAs are required for normal plant development.

To identify miRNAs that play a role in plant growth and development, genetic screens of miRNAs and/or their targets and computational prediction based on sequence complementarity between miRNAs and their target genes have been employed (3). These efforts revealed the existence of a number of miRNAs playing a critical role in leaf development (4), flower development (5), root development (6), developmental transition (7) and flowering (8). Importantly, the recently developed deep sequencing technologies have greatly improved the efficiency of finding novel miRNAs functioning under various conditions and from different plant species (9). The approaches have revealed that some miRNA families are well-conserved across plant species (10), suggesting that the regulatory role of many miRNAs may be universal in plants.

Ambient growth temperature (>15°C or non-stress temperature), which affects the rates of metabolic reactions

\*To whom correspondence should be addressed. Tel: +82 2 3290 3451; Fax: +82 2 927 9028; Email: jahn@korea.ac.kr

The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint first authors.

and morphogenetic processes, significantly influences plant growth and development (11). Flowering is the primary trait affected by the changes in ambient temperature (12–14). Genetic screens have identified a group of mutants whose flowering time is not affected by ambient temperature changes, thereby proposing a genetic pathway (thermosensory pathway) that mediates ambient temperature signaling (15). *FCA* (16) and *FVE* (17,18) act within the thermosensory pathway. More recently, the flowering-time repressors *short vegetative phase (SVP)* (19) and *flowering locus M (FLM)* (20) were identified as the key regulators of ambient temperature-responsive flowering (21,22). *SVP* negatively regulates the expression of *flowering locus T (FT)* (23,24) to exert its effect on flowering time by directly binding to the vCARG motifs in the *FT* promoter (22). Of particular interest, although *flowering locus C (FLC)* (25,26), an important floral repressor mediating the vernalization response, also negatively regulates *FT* expression via direct binding (27,28), the responses of *svp* and *flc* mutants to ambient temperature changes are different. This suggests that the signaling of ambient temperature (non-stress temperature) and vernalization (near-freezing temperature) may be distinct. Furthermore, an indirect relationship between light and ambient temperature signaling has been suggested by the temperature-independent flowering phenotypes of photoreceptors and *elf3* mutants (29,30). These findings suggest that ambient temperature signaling may be intricately interconnected to other signaling pathways (31).

Several plant miRNAs that play a role in the temperature response under stress conditions have been identified. By analysis of expressed sequence tags against known plant miRNAs, 25.8% of the miRNAs are predicted to be involved in a plant's response to biotic and abiotic stress, of which 19.0% are involved in the response to temperature stress (32). Deep sequencing analysis of a cold stress-treated small RNA library revealed that miR319, miR393, miR397 and miR402 are up-regulated and miR398 is down-regulated under cold stress (33). Transcriptome analysis based on the expression of miRNA targets also demonstrated that 11 miRNA families (miR156/157, miR159/319, miR164, miR165/166, miR169, miR172, miR393, miR394, miR396, miR397 and miR398) are induced or constantly expressed by cold treatment (34). However, the identification of temperature-responsive miRNAs so far has been performed mainly under stress temperature conditions; thus, the miRNA expression profile on changes of ambient temperature and the function of miRNAs under non-stress temperature conditions remain unknown.

In this study, we identified the ambient temperature-responsive miRNAs of *Arabidopsis*. To serve as a repository of these miRNAs, we also determined the expression profile of all unique miRNA loci in response to ambient temperature changes by miRNA northern hybridization analysis. We analyzed expression patterns of the ambient temperature-responsive miRNAs in the thermosensory pathway mutants and flowering-time phenotypes of miR172-overexpressing plants at two different ambient temperatures.

## MATERIALS AND METHODS

### Plant materials and growth conditions

Wild-type *Arabidopsis thaliana* plants (ecotype: Columbia) were grown in Sunshine Mix 5 (Sun Gro Horticulture, Quincy, MI, USA) or solidified Murashige and Skoog (MS) medium at 16 or 23°C under long day (LD; 16:8 h, light:dark) conditions with light supplied at an intensity of 120  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The seeds of *fve-3* and *fca-9* mutants were kindly provided by Dr Ilha Lee (Seoul National University, Seoul, Korea) and Dr Caroline Dean (John Innes Centre, Norwich, UK), respectively. The seeds were stratified at 4°C for 2 days and then transferred to soil or MS medium. Flowering time was measured by scoring the total number of primary rosette and cauline leaves of ~10–15 plants at bolting. All thermosensory pathway mutants used in this study were in the Columbia background. To generate miR172a-overexpressing plants (miR172a OX), a genomic fragment of miR172a was amplified by PCR and cloned into a destination vector harboring the 35S promoter using the Gateway system. The recombinant plasmid was introduced into wild-type Columbia plants using the floral dip method (35). Homozygous plants in the T<sub>3</sub> generation were used to analyze the flowering time and expression patterns.

### Total RNA isolation

For miRNA microarray analysis, total RNA was extracted by using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) from 10-day-old wild-type seedlings grown at 23 and 16°C under LD conditions. High-molecular-weight RNA species (>300 nucleotides in length) were removed by using a Microcon YM-100 centrifugal filter (Millipore Corp., Billerica, MA, USA). The flow-through containing small RNA species was collected by using Microcon YM-3 (Millipore). The enriched small RNAs were labeled with a Label IT miRNA labeling kit (Mirus Bio LLC, Madison, WI, USA) according to the manufacturer's instructions. For miRNA northern blot analysis, total RNA was extracted by using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), whereas for semiquantitative RT-PCR or quantitative real-time PCR (qRT-PCR), total RNAs were prepared by using TRIzol reagent (Invitrogen).

### miRNA microarray analysis

The miRNA microarray analysis was performed by using miRNA *Arabidopsis* 9.0 microarray (CombiMatrix Corp., Mukilteo, WA, USA). The microarray contains 87 *Arabidopsis* miRNA loci (Supplementary Table S1). The miRNA probe sequences were designed for complementarity with the full-length mature miRNAs listed in miRBase (<http://microrna.sanger.ac.uk>). A double mismatch probe (2mut) for each miRNA was designed to ensure specificity of detection and used as a negative control. The prepared RNA samples were mixed in hybridization buffer (12% formamide, 6 × SSPE buffer, 2.5% SDS and 0.8% BSA) at 37°C for 3 h. The chamber

was prehybridized using a buffer containing  $6 \times$  SSPE, 1% SDS and 0.2% BSA at 65°C for 10 min. Three successive washings were performed by using a washing solution ( $6 \times$  SSPET,  $3 \times$  SSPET and  $0.5 \times$  SSPET) at room temperature for 3 min. The microarray images scanned by Axon GenePix 4000B (Molecular Devices, Sunnyvale, CA, USA) were analyzed with the microarray imager application. Systematic variation affecting the measured expression levels was excluded from the raw data. The lowest 5% of all signals including perfect matches and mismatches was considered background signals. The data obtained from the arrays were normalized by using global scale factors to bring all backgrounds to the same value. All perfect matches that did not satisfy this criterion were converted to the background value, and all information on 2mut mismatches was discarded. Probes having signal values exceeding the background levels by factors of at least two were used for further analysis.

### miRNA northern hybridization analysis

To detect the mature forms of *Arabidopsis* miRNAs, an enhanced miRNA detection method using chemical cross-linking with 1-ethyl-3-(2-dimethylaminopropyl) carbodiimide (EDC) (36) or a method using conventional UV cross-linking was employed. We used the EDC method for northern hybridization analyses of 120 unique miRNA loci (Figure 2) and some miRNA loci showing weak signal intensities (Figure 4A), because our preliminary experiment using miR156a as a probe revealed that the EDC method was  $\sim 100$  times more sensitive than the conventional UV cross-linking method (Supplementary Figure S1). However, other miRNA northern hybridization analyses (Figures 3 and 5B) were performed using conventional UV cross-linking method. Before we conducted northern hybridization, we tested the probe specificity, because miRNAs are only 20–24 nucleotides long and often closely related. These data are shown in Supplementary Figure S2.

For northern hybridization analysis of 120 unique miRNA loci, 8-day-old wild-type plants grown under LD conditions at 23 or 16°C were harvested at two time points [zeitgeber 8 (ZT8) and ZT10]. Total RNA (10  $\mu$ g) was loaded onto 17% denaturing polyacrylamide gel containing 7M urea and electrophoresed. Separated total RNA was transferred to Hybond-NX neutral nylon membrane (GE Healthcare) using a Trans-Blot SD semi-dry transfer cell apparatus (Bio-Rad Laboratories, Hercules, CA, USA). To design the hybridization probes, *Arabidopsis* miRNA sequences were obtained from the *Arabidopsis* Small RNA Project website (<http://asrp.cgrb.oregonstate.edu>) (37), and 120 unique mature sequences of *Arabidopsis* miRNAs were chosen (Supplementary Table S1). The single-stranded DNA oligonucleotides complementary to the 120 unique sequences were labeled at the 3'-end with [ $\gamma$ - $^{32}$ P] ATP by using OptiKinase (USB Corp., Cleveland, OH, USA). Radiolabeled probes were then purified by using a Micro Bio-Spin P-6 column (Bio-Rad) to remove unincorporated radioisotopes. The radioactive probes were hybridized with transferred membranes in a

PerfectHyb buffer solution (Sigma-Aldrich, St. Louis, MO, USA) overnight at 42°C. The hybridized membranes were washed thrice in a washing buffer ( $2 \times$  SSC, 0.2% SDS) at 50°C for 5, 20 and 20 min. A second washing ( $1 \times$  SSC, 0.1% SDS) was carried out twice at 50°C for 20 min each.

To quantify miRNA expression levels, either U6 RNA levels or ethidium bromide-stained rRNAs was used. We used rRNA for a quantification control in the small RNA blots obtained using EDC cross-linking methods (Figures 2 and 4A), because U6 RNA is not suitable for a quantification control in the EDC blots (38). For the small RNA blots produced using conventional UV cross-linking method (Figures 3 and 5B), U6 RNA was used as a quantification control. Two independent northern hybridization assays with biological replicates (RNAs derived from distinct plant materials) were conducted.

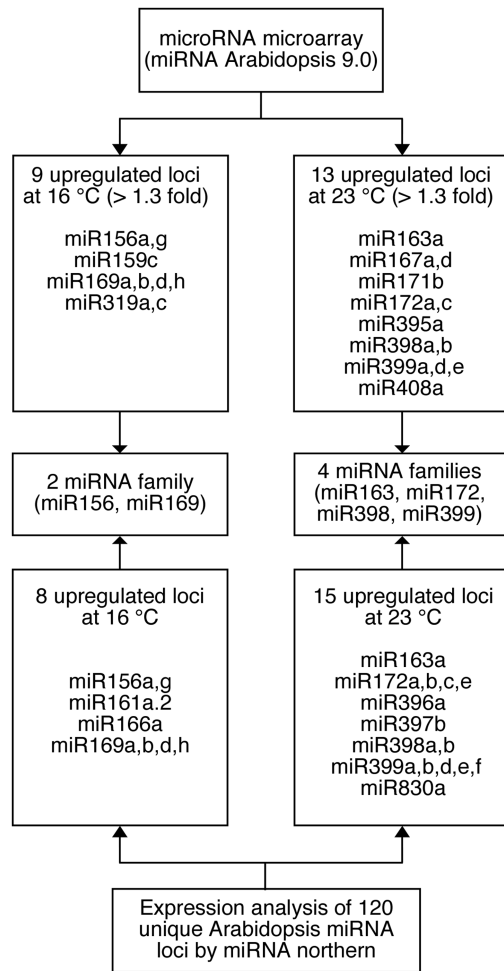
### qRT-PCR and RT-PCR analyses

To measure the expression levels of miRNA target genes and flowering-time genes, either qRT-PCR or RT-PCR analysis was used. For qRT-PCR, total RNAs with high-quality ( $A_{260}/A_{280} > 1.8$  and  $A_{260}/A_{230} > 2.0$ ) were used. Total RNA (1  $\mu$ g) treated with DNaseI (New England Biolabs, Inc., Ipswich, MA, USA) was used for synthesizing first-stranded cDNA with the oligo-dT primer using reverse transcriptase (Roche, Basel, Germany) in accordance with the manufacturer's instructions. qRT-PCR was performed using LightCycler 480 system (Roche Applied Science, Indianapolis, IN, USA) and SYBR Green PCR master mixture (Roche, Basel, Germany). Real-time PCR amplification efficiency of our qRT-PCR primers was at least 1.8. At least two biological replicates with two or three technical replicates were performed with similar results. Results from one biological replicate are presented. About 30 seedlings were used for each replicate. For qRT-PCR and RT-PCR analyses, expression was normalized against *Tubulin* and *UBQ10* genes, respectively. Sequence information of the oligonucleotides used for qRT-PCR and RT-PCR analyses in this study is available in Supplementary Table S2.

## RESULTS

### Identification of ambient temperature-responsive miRNAs

To identify the *Arabidopsis* miRNAs whose expression levels were altered by the changes in ambient temperature, we first performed a microarray analysis using the CombiMatrix *Arabidopsis* miRNA array. From the microarray results, we selected the miRNAs whose expression levels increased or decreased by more than 1.3-fold (Figure 1), and found that 22 miRNA loci corresponding to 12 miRNA families satisfied this criterion. The remaining miRNA loci did not show significant changes in their expression or were considered undetectable (absence). Fold change values of the miRNA loci tested in this experiment are listed in



**Figure 1.** Outline of the identification of the ambient temperature-responsive miRNAs in *Arabidopsis*. The miRNAs consistently identified by both the miRNA microarray and northern hybridization analyses were grouped and named as ambient temperature-responsive miRNAs.

Supplementary Table S3. The expression of nine miRNA loci (miR156a, g; miR159c; miR169a, b, d, h; miR319a, c) was up-regulated at 16°C and that of 13 miRNA loci (miR163a; miR167a, d; miR171b; miR172a, c; miR395a; miR398a, b; miR399a, d, e; miR408a) was up-regulated at 23°C.

Table 1 lists the fold changes of the miRNA loci selected from the microarray experiment. Among the miRNAs showing up-regulated expression at 16°C, miR169a showed the highest fold change (1.56). Notably, the expression of all known miR169 loci (miR169a, b, d, h) was elevated and satisfied the criterion, suggesting that the up-regulation of miR169 at 16°C was reproducible. In contrast, not all loci were selected in the case of miR156, miR159 and miR319. One locus of miR156 (miR156h) and two loci of miR159 (miR159a, b) did not satisfy the criterion. Among the miRNAs showing up-regulated expression at 23°C, miR398a showed the highest fold change (1.97). All miR398 loci (miR398a, b) showed increased expression at 23°C, similar to that seen in miR169 at 16°C. In the case of miR172, miR167 and

**Table 1.** The miRNA loci showing altered expression levels in the miRNA microarray analysis

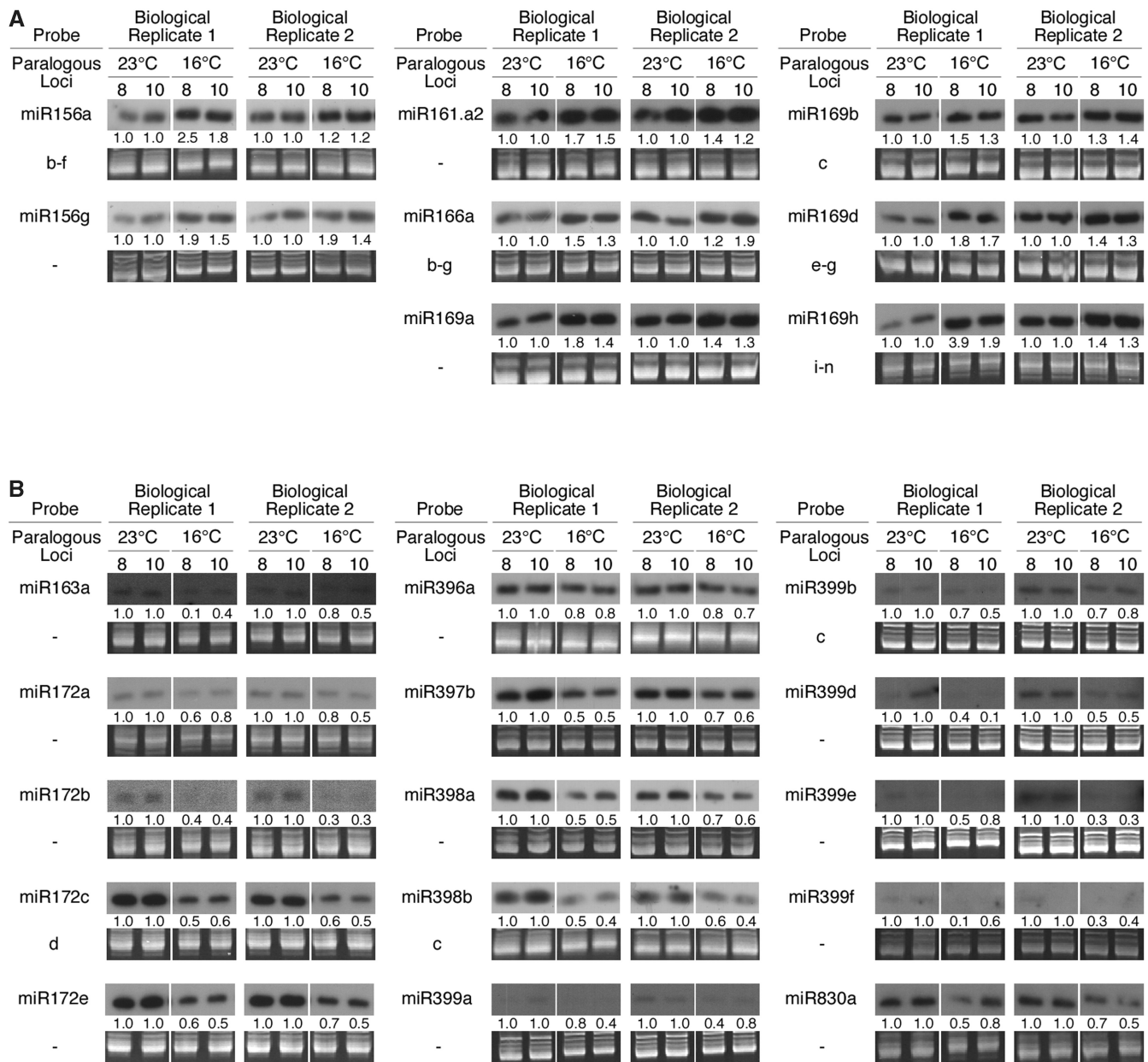
Temperature	Up-regulated miRNA locus	Paralogous locus/loci with identical sequence	Expression level change (>1.3-fold)
16°C	miR169a		1.56
	miR156g		1.47
	miR169b	c	1.46
	miR319c		1.41
	miR169h	i, j, k, l, m, n	1.37
	miR156a	b, c, d, e, f	1.36
	miR159c		1.35
	miR169d	e, f, g	1.34
	miR319a	b	1.32
	miR398a		1.97
23°C	miR398b	c	1.88
	miR163a		1.61
	miR172c	d	1.53
	miR171b	c	1.41
	miR167d		1.36
	miR408a		1.35
	miR395a	d, e	1.33
	miR172a	b	1.32
	miR167a	b	1.32
	miR399d		1.31
miR399e		1.31	
miR399a		1.30	

miR399, not all loci of each miRNA showed more than 1.3-fold change; however, as miR172e; miR171a, c; miR167c; and miR395b, c, f did not satisfy the criterion, they were not selected in our microarray experiment. Together, our microarray analysis revealed that the expression of a subset of *Arabidopsis* miRNAs was altered by the changes in ambient temperature.

#### Expression levels of the 120 unique miRNA loci in response to ambient temperature changes

The miRNA microarray analysis allowed us to quickly compare the miRNA expression levels at 23 and 16°C; however, the experiment had some limitations, such as incomplete coverage of known *Arabidopsis* miRNAs and possible false results after normalization. Therefore, to complement the miRNA microarray analysis and build a comprehensive repository of information regarding the ambient temperature-responsive miRNAs, we measured the expression levels of all unique miRNA loci in *Arabidopsis* by enhanced miRNA northern hybridization analysis (36).

The analysis revealed 70 miRNA loci showing detectable signals (Figure 2). The remaining 50 miRNAs, mostly miR700 and miR800 series, were barely detectable (Supplementary Figure S3), suggesting that these undetected miRNAs were of low abundance in the developmental stage of the wild-type plants tested or that our miRNA northern analysis had limited sensitivity. This analysis demonstrated that four miRNAs (miR156a, g; miR161.a2; miR166a; miR169a, b, d, h) were up-regulated at 16°C (Figure 2A) and seven miRNAs (miR163a; miR172a, b, c, e; miR396a; miR397b; miR398a, b; miR399a, b, d, e, f; miR830a) were up-regulated at 23°C (Figure 2B). The miRNAs that did



**Figure 2.** miRNA northern hybridization analysis of unique *Arabidopsis* miRNA loci with two biological replicates. The numbers above the blots indicate the times of harvest (ZT). The numbers below each miRNA blot denote fold change relative to the miRNA level at 23°C. Ethidium bromide-stained rRNAs are shown to demonstrate an equal amount of loading. Note that a probe hybridizes to its paralogous loci as well as itself, because of their identical sequences. The miRNA loci showing up-regulation at 16°C (A) and 23°C (B) are shown.

not show significant expression level changes or that showed inconsistent results in the biological replicates or between the different ZT time points are shown in Supplementary Figure S4. This northern hybridization analysis revealed that all miR169 loci (miR169a, b, d, h) and all miR172 loci (miR172a, b, c, e) were up-regulated at 16 and at 23°C, respectively. None of the *Arabidopsis* miRNA loci were found to be up-regulated under both temperature conditions.

Comparison of the results obtained from the microarray and northern hybridization analyses revealed that there are 14 overlapped miRNA loci, corresponding to six

miRNA families (two up-regulated 16°C and four up-regulated at 23°C; Figure 1). Therefore, we chose these six miRNAs (miR156, miR163, miR169, miR172, miR398 and miR399) for high-fidelity selection, named them ambient temperature-responsive miRNAs, and performed further studies.

#### Anticorrelated expression patterns of the target genes of the ambient temperature-responsive miRNAs at 23 and 16°C

To assess whether the target genes of six ambient temperature-responsive miRNAs (Table 2) were

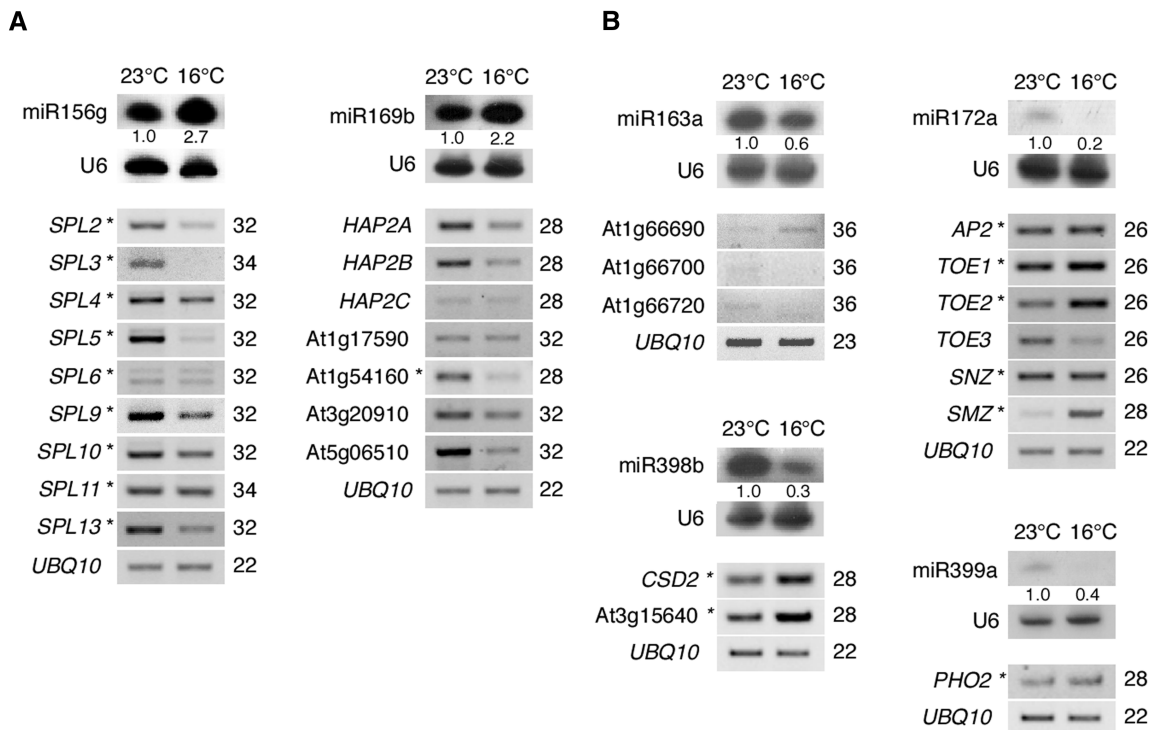
down-regulated under the conditions where the miRNAs were up-regulated, we examined the transcript levels of their target genes in wild-type plants grown at 23 and 16°C via RT-PCR. If anticorrelated expression patterns between these miRNAs and their target genes are found, it is reasonable to assume that up-regulated miRNAs at 23 or 16°C negatively regulate their target genes at each temperature.

The RT-PCR analysis revealed that, among the target genes of miR156, the expression of *SQUAMOSA PROTEIN BINDING PROTEIN LIKE2 (SPL2)*, *SPL3*, *SPL4*, *SPL5*, *SPL9*, *SPL10* and *SPL13* was significantly down-regulated at 16°C (Figure 3A), the temperature at which miR156 expression was enhanced. In the case of

miR169, the expression of *Heme Activator Protein 2A (HAP2A)*, *HAP2B*, *At1g54160* and *At5g06510* was significantly down-regulated at 16. At 23°C, the expression of *At1g66690* (miR163 target gene) was down-regulated whereas those of *At1g66700* and *At1g66720* were weakly increased. Two other targets, *At3g44860* and *At3g44870*, were undetectable even after extended RT-PCR cycles (data not shown). Among the six target genes of miR172, the expression levels of *target of EAT2 (TOE2)* and *SCHLAFMÜTZE (SMZ)* were significantly reduced at 23°C (Figure 3B). In the case of the miR398 target genes, expression of *Copper/Zinc Superoxide Dismutase 2 (CSD2)* and *At3g15640* was down-regulated at 23°C. In the case of miR399, the

**Table 2.** The ambient temperature-responsive miRNAs and the functions of their target genes

miRNA	Number of loci	Target family	Number of targets	Function
miR156	12	SPL	11	Flowering time, vegetative phase change (60,62–65)
miR163	1	SAMT	5	Defense (39,40)
miR169	14	HAP2	7	Flowering time, drought stress (61,66,67)
miR172	5	AP2	6	Flowering time, floral organ identity (5,8,42,43)
miR398	3	CSD2	2	Oxidative stress tolerance (68,69)
miR399	6	PHO2	1	Phosphate ion homeostasis (58,70)



**Figure 3.** Expression of the six ambient temperature-responsive miRNAs and their target genes at 23 and 16°C. Total RNAs isolated from 10-day-old wild-type seedlings and the same RNA were used for miRNA northern hybridization and RT-PCR of the target genes. The RT-PCR results are presented under the respective panels of the miRNA expression. (A) Expression of miR156 and miR169, showing up-regulation at 16°C, and the transcript levels of their target genes. (B) Expression of miR163, miR172, miR398 and miR399, showing up-regulation at 23°C, and the transcript levels of their target genes. The numbers below the miRNA blots denote fold change relative to the miRNA level at 23°C. The target genes whose cleavage site was validated by 5' Rapid amplification of complementary DNA ends (RACE-PCR) analysis (8,43,44,58–60) or target genes whose expression was down-regulated in transgenic plants overexpressing miRNA (44,61) are indicated by an asterisk. The numbers to the right of every panel indicate the number of PCR cycles. U6 RNA and *UBQ10* were used for a control for northern hybridization and RT-PCR analyses, respectively.

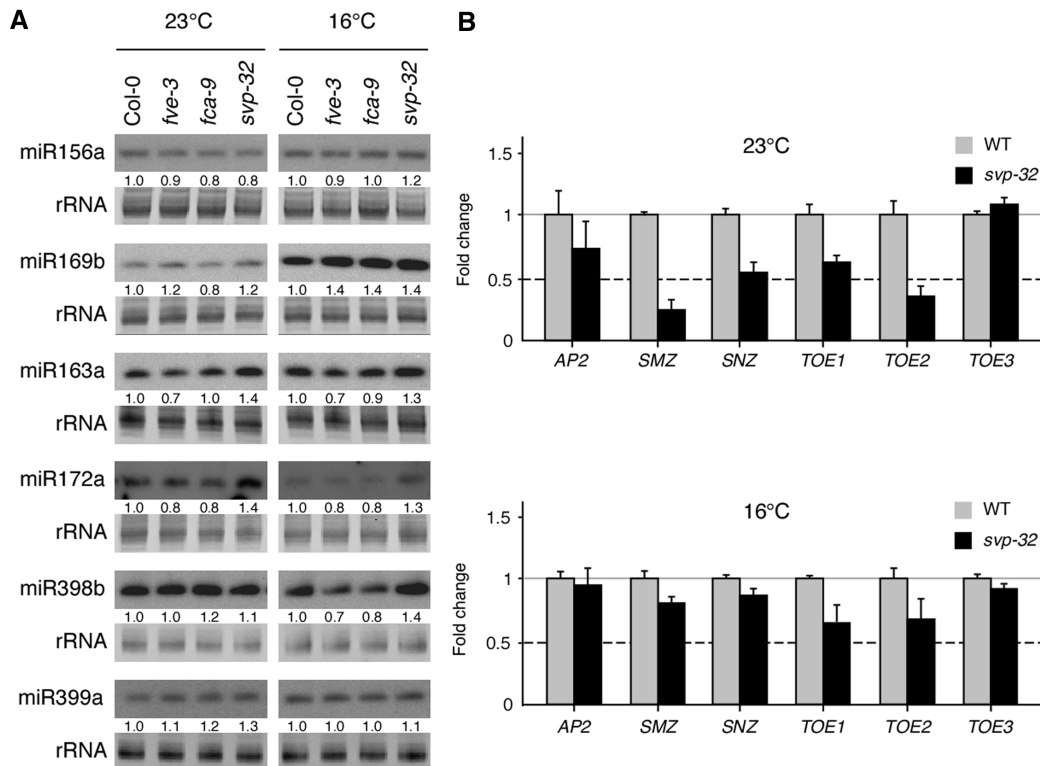
expression of its single target, *PHO2*, was weakly diminished at 23°C. Taken together, of the 32 ambient temperature-responsive miRNA target genes, the expression of 17 genes was negatively correlated in response to the changes in ambient temperature. This analysis revealed that the expression levels of the ambient temperature-responsive miRNAs and their target genes were largely anticorrelated, at least at the transcript level, and raised the possibility that ambient temperature signaling via these miRNAs may be selectively mediated by a subset of their target genes.

**Expression patterns of the ambient temperature-responsive miRNAs and their target genes in the thermosensory pathway mutants**

The timing of flowering in response to ambient temperature changes is regulated by the thermosensory pathway genes (15,22). To assess whether the ambient temperature-responsive miRNAs identified in this study play a role in thermosensory signaling, the expression patterns of these miRNAs were analyzed in thermosensory pathway mutants (*fve-3*, *fca-9* and *svp-32*).

miRNA northern hybridization analysis demonstrated that the expression levels of some ambient temperature-responsive miRNAs were affected in the *svp-32* mutants (Figure 4A), whereas changes in the miRNA expression were weak in the *fve-3* and *fca-9* mutants. Expression of miR156a was not significantly

altered in the thermosensory pathway mutants at 23 and 16°C, suggesting that miR156 does not act downstream of these genes. Compared with wild-type plants, slightly increased expression of miR169b was seen in the thermosensory pathway mutants at 16°C, whereas no significant alteration was observed at 23°C. The similar increase in miR169b expression at 16°C was likely irrelevant to flowering-time control, because the flowering-time phenotypes of the *fca*, *fve* and *svp* mutants were very distinct. miR163a expression was slightly reduced in the *fve-3* mutants but increased in the *svp-32* mutants at both temperatures. Considering that *S-adenosylmethionine-dependent methyltransferase (SAMT)* genes play a role in plant defense (39,40) and salicylic acid (mediating plant defense responses) affects flowering time (41), this observation suggested that the *fve-3* and *svp-32* mutants may have a differential response to the biotic and abiotic stress conditions. Expression of miR172a was increased in the *svp-32* mutants at both temperatures but remained unaltered in the *fca-9* and *fve-3* mutants. It was likely that the increase in miR172a expression in the *svp-32* mutants led to down-regulation of its target genes having floral repressor activities (42,43), explaining the early flowering phenotype of *svp-32* mutants (19,22). Expression of miR398b was slightly reduced in the *fve* and *fca* mutants but increased in the *svp-32* mutants at 16°C; however, its expression remained unaltered in the thermosensory pathway mutants at 23°C. The distinct



**Figure 4.** (A) Expression of six ambient temperature-responsive miRNAs in the thermosensory pathway mutants at 23 and 16°C. miRNA accumulation in the *fve-3*, *fca-9* and *svp-32* mutants grown for 10 days was measured by northern hybridization. Ethidium bromide-stained rRNAs were used as loading controls. The numbers below the blots denote the fold change relative to wild-type plants. (B) qRT-PCR analysis of the target genes of miR172 in 10-day-old *svp-32* mutants grown under LD conditions at 23 and 16°C. *Tubulin* was used to normalize the expression of target genes. Error bars indicate standard deviation.

changes in miR398b expression suggested that the thermosensory pathway mutants might have copper homeostasis-related phenotypes. Expression of miR399a was not significantly changed in the thermosensory pathway mutants at 23 and 16°C. Taken together, the miRNA northern hybridization analysis revealed that a lesion in *SVP* led to the changes in the expression levels of some of the ambient temperature-responsive miRNAs.

As the expression levels of miR172, known to regulate flowering time (8,42,43), were significantly altered in the *svp-32* mutants (Figure 4A), we analyzed the expression of its target genes in the *svp-32* mutants to determine whether their expression levels are consistently anticorrelated. qRT-PCR analysis revealed that the transcript levels of a subset of miR172 target genes were affected in the *svp-32* mutants at both temperatures. The expression levels of *APETALA2* (*AP2*), *SMZ*, *SCHNARCHZAPFEN* (*SNZ*), *TOE1* and *TOE2* were significantly reduced at 23°C in the *svp-32* mutants (Figure 4B). The expression of *TOE1* and *TOE2* was also decreased at 16°C, suggesting that the expression levels of miR172 and its target genes were consistently changed in the *svp-32* mutants at lower temperature. In summary, altered expression of miR172 and its target genes was seen in the *svp* mutants, suggesting a link between *SVP* and miR172 in the control of flowering time.

#### Overexpression of miR172 and temperature insensitivity

To determine whether miR172 functions in flowering-time control by ambient temperature, we tested the effect of this miRNA on the temperature response by overexpression. miR172a-overexpressing plants (Figure 5A and B) showed early flowering at 23°C (5.0 leaves, wild-type plants = 13.0 leaves; Figure 5C), consistent with the previous findings (8,42). Interestingly, the miR172a-overexpressing plants showed strong early flowering phenotype at 16°C (5.6 leaves, wild-type plants = 25.8 leaves). Thus, the flowering time of these plants was almost identical at both temperatures, revealing that miR172 overexpression led to temperature insensitivity in *Arabidopsis* and suggesting that modulation of an ambient temperature-responsive miRNA activity changes a plant's response to ambient temperature changes.

To obtain further insight into how miR172 regulates its targets by the changes in ambient temperature, the expression levels of its target genes were monitored in miR172a-overexpressing plants grown at 23 and 16°C under LD conditions. qRT-PCR analysis demonstrated that the expression levels of a subset of miR172 target genes were up-regulated in wild-type plants at 16°C (Supplementary Figure S5), consistent with low-level miR172 expression at 16°C in wild-type plants (Figures 3B and 4A). In the miR172a-overexpressing plants, the expression levels of *TOE2* and *SMZ* were significantly down-regulated at both 23 and 16°C (Figure 5D). *TOE1* expression was weakly down-regulated at 23 and 16°C. This result suggests that the flowering-time changes in response to ambient temperature changes by miR172 were largely mediated by *TOE1*, *TOE2* and *SMZ*. In contrast, the transcript levels

of *AP2*, *SNZ* and *TOE3* were slightly increased or unaltered in the miR172a-overexpressing plants. The unaltered expression level of *AP2* was consistent with a previous finding (44). Taking into consideration the observation that the expression levels of miR172 and its target genes were significantly affected in the *svp* mutants (Figure 4), these results suggested that the miR172 pathway in response to ambient temperature changes may be, at least partly, dependent on the thermosensory pathway.

To better understand the molecular mechanisms by which miR172 controls the temperature response at 23 and 16°C, we investigated the expression levels of flowering-time genes in miR172a-overexpressing plants. qRT-PCR analysis showed that the level of *FT* expression (23,24) was markedly increased at both temperatures (Figure 5E), providing a molecular basis explaining why the miR172a-overexpressing plants were early flowering. However, the transcript levels of *twin sister of FT* (*TSF*) (45,46), a close homolog of *FT* that also acts as a floral activator, remained unaffected in these plants, suggesting that the ambient temperature response is mainly mediated by *FT*. In addition, the expression of *suppressor of overexpression of constans 1* (*SOCI*) (47,48) was not altered at both temperatures, which was consistent with a previous finding (42). Interestingly, the expression of *FLC*, which negatively regulates the expression of *FT* via direct binding to the CArG box in these genes (27,28), was reduced in the miR172a-overexpressing plants. *SVP* expression was not affected in these plants, consistent with a role of miR172 downstream of *SVP* (Figure 4). The expression of *API*, a molecular marker for floral transition, was precociously detected, consistent with the early flowering phenotype of the miR172a-overexpressing plants. These results indicated that the early flowering phenotypes observed on miR172a overexpression at 23 and 16°C are caused by up-regulation of *FT*, possibly via the repression of *FLC*.

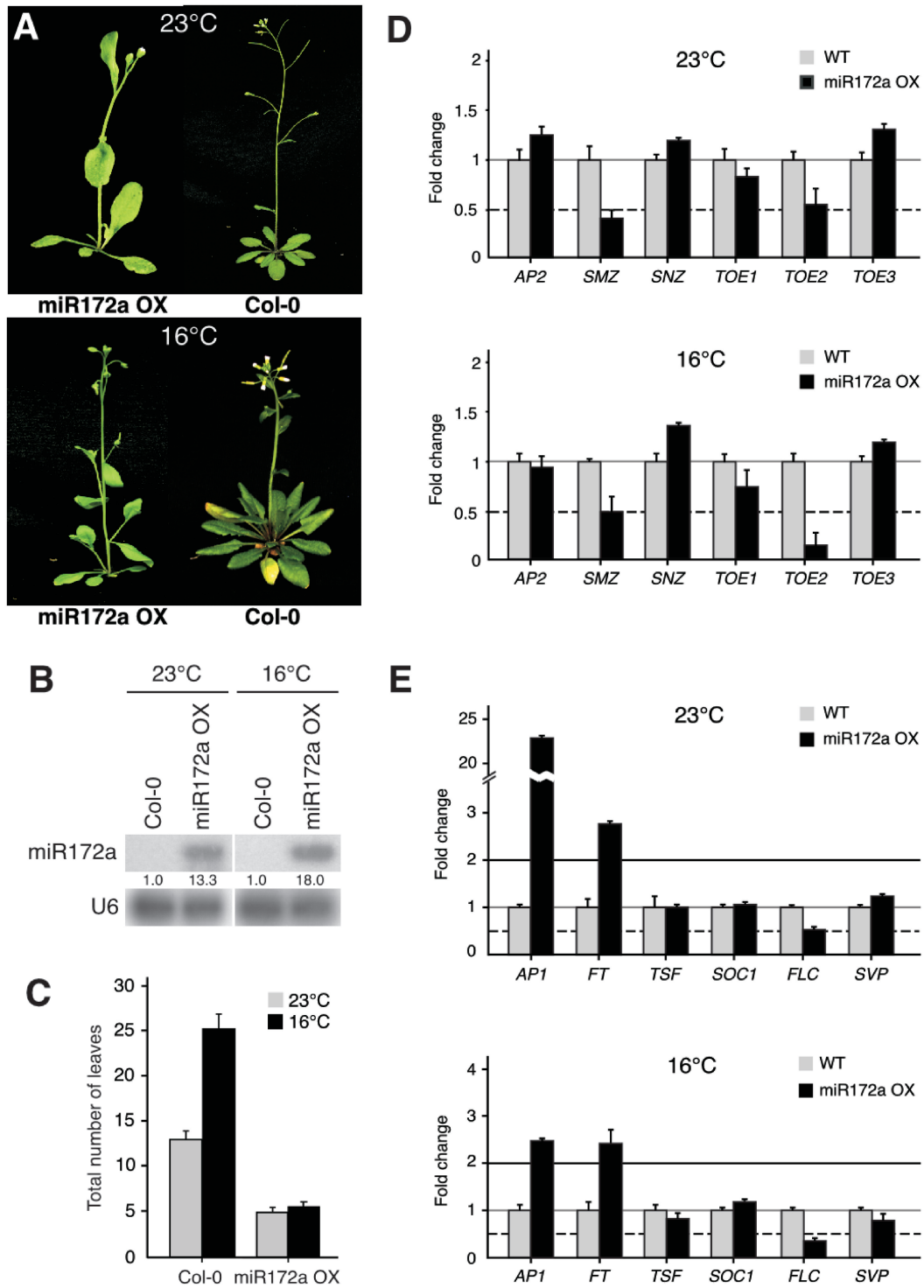
## DISCUSSION

In this study, we identified six ambient temperature-responsive miRNAs and found anticorrelated expression patterns between the miRNAs and their target genes at two different ambient temperatures. We suggest that *SVP* may function as a link between miR172 and the thermosensory pathway. Finally, we showed that miR172 overexpression changes the plant response to ambient temperature changes.

#### Identification of the ambient temperature-responsive miRNAs

Recently, microarrays have been widely used to perform high-throughput profiling of the expression of miRNAs, especially under stress or differential environmental conditions in plants (49,50). In this study, to overcome the limitation of the microarray-based approach and build a repository of information, we performed miRNA northern hybridization analysis after miRNA microarray analysis by using 120 unique miRNA loci as probes.





**Figure 5.** Changes in the ambient temperature response by miR172 overexpression. Phenotype (A) and flowering time (C) of miR172a-overexpressing plants (miR172a OX) grown under LD conditions at 23 and 16°C. The error bars denote the standard deviation. (B) miRNA northern hybridization to show overproduction of miR172 in miR172a OX plants grown at 23 and 16°C. (D) qRT-PCR analysis of the target genes of miR172 in 8-day-old miR172a OX plants grown under LD conditions at 23 and 16°C. (E) qRT-PCR analysis of flowering-time genes in 8-day-old miR172a OX plants grown under LD conditions at 23 and 16°C.

Comparison of the results obtained from both approaches demonstrated largely consistent expression changes of the miRNAs in response to changes in ambient temperature. However, it should be noted that 17 miRNA loci were identified in either one of the analyses. A possible

explanation for this partial overlap is that we used a high threshold level for the selection of ambient temperature-responsive miRNAs from the microarray. If our selection criterion is decreased to 1.2-fold, some miRNAs [miR399f (1.28) and miR396a (1.25)] were

included in the final set. Another explanation is the limited sensitivity and reproducibility of small RNA northern hybridization analysis, although sensitivity was significantly increased by employing the EDC cross-linking method. Some miRNAs (miR159, miR167 and miR408) showing high fold change in microarray analysis did not show significant differences in northern hybridization. These problems regarding sensitivity and reproducibility of two different techniques used for profiling miRNA expression have been recently addressed (51). To resolve this discrepancy, we are currently examining the changes in miRNA abundance using deep sequencing technologies.

Despite the combined approach, a limitation of our study is that we selected only those miRNAs whose target genes were transcriptionally affected; therefore, the target genes inhibited at the translational level were excluded. There may be more miRNAs that are responsive to ambient temperature changes. This assumption is possible partly because the changes in ambient temperature caused a wide range of physiological effects in the plants, which cannot be explained by the target genes of the ambient temperature-responsive miRNAs identified in this study alone. This possibility is supported by the recent proposal that translational repression of targets by miRNAs is a general mechanism in plants (52). Therefore, we cannot conclude that the ambient temperature-responsive miRNAs identified in this study are the only miRNAs that respond to ambient temperature changes. Screens of miRNA targets subjected to translational repression will be required to unveil another level of complexity in the ambient temperature-regulated response.

#### Most ambient temperature-responsive miRNAs are a subset of cold stress-responsive miRNAs

Comparison of our results with those previously obtained following abiotic stress treatment (33,34,49,50) revealed that miR156, miR169, miR172 and miR398 are cold stress-responsive miRNAs (Table 3). Among the cold stress-responsive miRNAs, 14 miRNAs (miR157, miR159, miR164, miR165, miR166, miR168, miR171, miR319, miR393, miR394, miR396, miR397, miR400 and miR408) are not included in ambient temperature-responsive miRNAs. Meanwhile, only two miRNAs (miR163 and miR399) are unique miRNAs that were not identified under abiotic stress conditions including cold. This partial overlap between the ambient temperature-responsive miRNAs and cold stress-responsive miRNAs suggests that ambient temperature signaling and cold stress signaling may partially overlap, at least at the level of miRNA-mediated gene regulation. However, the incomplete overlap also suggests that the plant response to different temperature ranges is mediated by a different set of miRNAs. If this is true, it is probable that the 14 miRNAs not included in the ambient temperature-responsive miRNAs play a preferential role in signaling of near-freezing temperature and the overlapping miRNAs play a role over a broad range of

**Table 3.** The *Arabidopsis* miRNAs involved in abiotic stress and ambient temperature response

miRNA	Salinity (49)	Drought (34)	UV (71)	Cold (33,34)	Ambient temperature (this study)
miR156	○		○	○	○
miR157		○	○	○	
miR158	○				
miR159	○		○	○	
miR163					○
miR164				○	
miR165	○		○	○	
miR166			○	○	
miR167	○	○	○		
miR168	○	○		○	
miR169	○			○	○
miR170			○		
miR171	○	○	○	○	
miR172			○	○	○
miR319	○		○	○	
miR393	○	○	○	○	
miR394	○			○	
miR395					
miR396	○	○		○	
miR397				○	
miR398			○	○	○
miR399					○
miR400				○	
miR401			○		
miR408		○		○	

temperatures. Further research is required to elucidate the functional divergence of miRNAs in a wide range of temperature responses.

An important question is whether genes acting in miRNA biogenesis are involved in the ambient temperature response, because some mutants (*hen1-1* and *dcl1-9*) impaired in the miRNA biogenesis process show altered sensitivity to abiotic stress (33). However, unlike abiotic stress-related miRNAs, several lines of evidence suggest that proteins acting in miRNA biogenesis do not function in the ambient temperature response. First, *Arabidopsis* plants grown at the low temperature did not exhibit the altered phenotypes seen in the *dcl1* and *ago1* mutants, although the leaf morphology and appearance was slightly altered (13). Second, the miRNA biogenesis mutants responded normally to the changes in ambient temperature, with flowering-time ratios (16/23°C) ranging from 1.9 to 2.4 (Supplementary Figure S6). Third, the expression of miR162, miR168 and miR403, targeting *DCL1*, *AGO1* and *AGO2* (53–55), was not changed by the ambient temperature (this study). Consistent with this observation, the expression levels of some miRNA biogenesis genes were not altered at 16 and 23°C (Supplementary Figure S7).

#### *SVP* may act as an upstream mediator of miR172 in the control of flowering time

We showed that loss of *SVP* function alters the miR172 expression level (Figure 4). The altered expression of miR172 in the *svp* mutants suggests that *SVP* is an

upstream mediator of miR172 in the ambient temperature response. It is likely that *SVP*-miR172 signaling is ultimately integrated by *FT*, an output gene of the thermosensory pathway. This hypothesis is supported by the observations that early flowering of the miR172-overexpressing plants at 23 and 16°C resulted from the up-regulation of *FT* expression independently of *SVP* (Figure 5). Considering that *SVP* binds to its target site known as CARG motifs (22,56), it is possible that miR172 transcription is also regulated by direct binding of *SVP* within the miR172 loci. This notion is consistent with the observation that several canonical CARG or variants of CARG boxes can be found within the miR172 loci (from -2602 to -2593 and -968 to -959 in miR172a; from -1240 to -1231, -700 to -691 and -423 to -414 in miR172b; from -1429 to -1420, -1078 to -1069 and -866 to -857 in miR172c; from -820 to -811 in miR172e). However, the *SVP*-miR172 interaction in ambient temperature signaling could be minor, compared with the *SVP*-*FT* interaction, because a major role of *SVP* seems to be the regulation of *FT* and *TSF* expression (22,57) and because increased expression of miR172 in the *svp* mutants may be insufficient for up-regulation of *FT*. Thus, it is probable that *SVP* regulates multiple downstream genes to fine-tune flowering time within the thermosensory pathway. Further analysis is required to elucidate the transcriptional regulation of miR172 by *SVP* in ambient temperature signaling.

### Overexpression of miR172 changes the plant's temperature sensitivity in response to ambient temperature changes

In this study, we have shown that loss of *SVP* function alters the expression of miR172 and its targets and that overexpression of miR172 causes temperature insensitivity (Figures 4 and 5). These results suggest that miR172 functions downstream of *SVP* within the thermosensory pathway. This notion is supported by the observation that overexpression of *SMZ* or *rSMZ* (a resistant version carrying mutations in the miR172-binding site) was completely suppressed the flowering phenotypes of *svp* mutants (43). Interestingly, overexpression of miR172 not only induced up-regulation of *FT* but also reduced *FLC* expression (Figure 5E). Considering that *FLC* expression remains unaltered by overexpression of *TOE1* and *SMZ*, the major target genes of miR172 (42,43), it is likely that *FLC* is indirectly regulated by miR172 target genes in response to ambient temperature changes. miR172 expression is known to be regulated in a temporally specific manner (8), implying that miR172 may sense plant developmental age. This notion is consistent with the observations that miR172 transcript levels were affected by some autonomous pathway mutants (42). Thus, it is probable that the reduced *FLC* expression may be due to the accelerated developmental stage of the miR172-overexpressing plants and not due to the changes in ambient temperature. It will be interesting to determine the possible role of *FLC* in the miR172-mediated signaling pathway.

Nonetheless, we cannot dismiss the possibility that the miR172-*FT* pathway may not be a major pathway *in vivo* in response to ambient temperature changes, since constitutive expression of miR172 may cause a broad effect and the overexpression phenotype may not reflect the situation that plants respond to ambient temperature changes *in vivo*. Further analysis of a knock out mutant of miR172 or a target mimicry line of miR172 is required to further elucidate a role of the miR172-*FT* pathway in plants.

In conclusion, our results suggest that miRNAs are involved in the ambient temperature response in *Arabidopsis* and that *SVP* may play an important role in regulating miR172 in the control of flowering time by ambient temperature changes. We propose that the *SVP*-miR172 regulatory circuit provides a fine-tuning mechanism in response to the ambient temperature changes, allowing plants to modify their development for successful adaptation under continuously fluctuating temperature conditions.

### SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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### REFERENCES

1. Carrington, J.C. and Ambros, V. (2003) Role of microRNAs in plant and animal development. *Science*, **301**, 336–338.
2. Schauer, S.E., Jacobsen, S.E., Meinke, D.W. and Ray, A. (2002) DICER-LIKE1: blind men and elephants in Arabidopsis development. *Trends Plant Sci.*, **7**, 487–491.
3. Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B. and Bartel, D.P. (2002) Prediction of plant microRNA targets. *Cell*, **110**, 513–520.
4. Palatnik, J.F., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J.C. and Weigel, D. (2003) Control of leaf morphogenesis by microRNAs. *Nature*, **425**, 257–263.
5. Chen, X. (2004) A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. *Science*, **303**, 2022–2025.
6. Guo, H.S., Xie, Q., Fei, J.F. and Chua, N.H. (2005) MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for Arabidopsis lateral root development. *Plant Cell*, **17**, 1376–1386.

7. Lauter, N., Kampani, A., Carlson, S., Goebel, M. and Moose, S.P. (2005) microRNA172 down-regulates *glossy15* to promote vegetative phase change in maize. *Proc. Natl Acad. Sci. USA*, **102**, 9412–9417.
8. Aukerman, M.J. and Sakai, H. (2003) Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. *The Plant Cell Online*, **15**, 2730.
9. Fahlgren, N., Howell, M.D., Kasschau, K.D., Chapman, E.J., Sullivan, C.M., Cumbie, J.S., Givan, S.A., Law, T.F., Grant, S.R., Dangel, J.L. *et al.* (2007) High-throughput sequencing of Arabidopsis microRNAs: evidence for frequent birth and death of MIRNA genes. *PLoS One*, **2**, e219.
10. Barakat, A., Wall, K., Leebens-Mack, J., Wang, Y.J., Carlson, J.E. and Depamphilis, C.W. (2007) Large-scale identification of microRNAs from a basal eudicot (*Eschscholzia californica*) and conservation in flowering plants. *Plant J.*, **51**, 991–1003.
11. Long, S.P. and Woodward, F.I. (1988) Company of Biologists and Society for Experimental Biology (Great Britain). *Plants and Temperature*. Company of Biologists Ltd., Department of Zoology, University of Cambridge, Cambridge, UK.
12. Penfield, S. (2008) Temperature perception and signal transduction in plants. *New Phytol.*, **179**, 615–628.
13. Lee, J.H., Lee, J.S. and Ahn, J.H. (2008) Ambient temperature signaling in plants: an emerging field in the regulation of flowering time. *J. Plant Biol.*, **51**, 321–326.
14. Samach, A. and Wigge, P.A. (2005) Ambient temperature perception in plants. *Curr. Opin. Plant Biol.*, **8**, 483–486.
15. Blazquez, M.A., Ahn, J.H. and Weigel, D. (2003) A thermosensory pathway controlling flowering time in Arabidopsis thaliana. *Nat. Genet.*, **33**, 168–171.
16. Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C. *et al.* (1997) *FCA*, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding domains. *Cell*, **89**, 737–745.
17. Kim, H.J., Hyun, Y., Park, J.Y., Park, M.J., Park, M.K., Kim, M.D., Kim, H.J., Lee, M.H., Moon, J., Lee, I. *et al.* (2004) A genetic link between cold responses and flowering time through FVE in Arabidopsis thaliana. *Nat. Genet.*, **36**, 167–171.
18. Ausin, I., Alonso-Blanco, C., Jarillo, J.A., Ruiz-Garcia, L. and Martinez-Zapater, J.M. (2004) Regulation of flowering time by FVE, a retinoblastoma-associated protein. *Nat. Genet.*, **36**, 162–166.
19. Hartmann, U., Hohmann, S., Nettesheim, K., Wisman, E., Saedler, H. and Huijser, P. (2000) Molecular cloning of SVP: a negative regulator of the floral transition in Arabidopsis. *Plant J.*, **21**, 351–360.
20. Scortecchi, K., Michaels, S.D. and Amasino, R.M. (2003) Genetic interactions between FLM and other flowering-time genes in Arabidopsis thaliana. *Plant Mol. Biol.*, **52**, 915–922.
21. Balasubramanian, S., Sureshkumar, S., Lempe, J. and Weigel, D. (2006) Potent induction of Arabidopsis thaliana flowering by elevated growth temperature. *PLoS Genet.*, **2**, e106.
22. Lee, J.H., Yoo, S.J., Park, S.H., Hwang, I., Lee, J.S. and Ahn, J.H. (2007) Role of SVP in the control of flowering time by ambient temperature in Arabidopsis. *Genes Dev.*, **21**, 397–402.
23. Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M. and Araki, T. (1999) A pair of related genes with antagonistic roles in mediating flowering signals. *Science*, **286**, 1960–1962.
24. Kardailsky, I., Shukla, V.K., Ahn, J.H., Dagenais, N., Christensen, S.K., Nguyen, J.T., Chory, J., Harrison, M.J. and Weigel, D. (1999) Activation tagging of the floral inducer FT. *Science*, **286**, 1962–1965.
25. Michaels, S.D. and Amasino, R.M. (1999) FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell*, **11**, 949–956.
26. Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J. and Dennis, E.S. (1999) The FLM MADS box gene: a repressor of flowering in Arabidopsis regulated by vernalization and methylation. *Plant Cell*, **11**, 445–458.
27. Searle, I., He, Y., Turck, F., Vincent, C., Fornara, F., Krober, S., Amasino, R.A. and Coupland, G. (2006) The transcription factor FLC confers a flowering response to vernalization by repressing meristem competence and systemic signaling in Arabidopsis. *Genes Dev.*, **20**, 898–912.
28. Helliwell, C.A., Wood, C.C., Robertson, M., James Peacock, W. and Dennis, E.S. (2006) The Arabidopsis FLC protein interacts directly *in vivo* with *SOC1* and *FT* chromatin and is part of a high-molecular-weight protein complex. *Plant J.*, **46**, 183–192.
29. Halliday, K.J., Salter, M.G., Thingnaes, E. and Whitelam, G.C. (2003) Phytochrome control of flowering is temperature sensitive and correlates with expression of the floral integrator FT. *Plant J.*, **33**, 875–885.
30. Strasser, B., Alvarez, M.J., Califano, A. and Cerdan, P.D. (2009) A complementary role for ELF3 and TFL1 in the regulation of flowering time by ambient temperature. *Plant J.*, **58**, 629–640.
31. Franklin, K.A. (2009) Light and temperature signal crosstalk in plant development. *Curr. Opin. Plant Biol.*, **12**, 63–68.
32. Zhang, B.H., Pan, X.P., Wang, Q.L., Cobb, G.P. and Anderson, T.A. (2005) Identification and characterization of new plant microRNAs using EST analysis. *Cell Res.*, **15**, 336–360.
33. Sunkar, R. and Zhu, J.K. (2004) Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. *Plant Cell*, **16**, 2001–2019.
34. Zhou, X., Wang, G., Sutoh, K., Zhu, J.K. and Zhang, W. (2008) Identification of cold-inducible microRNAs in plants by transcriptome analysis. *Biochim. Biophys. Acta*, **1779**, 780–788.
35. Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J.*, **16**, 735–743.
36. Pall, G.S., Codony-Servat, C., Byrne, J., Ritchie, L. and Hamilton, A. (2007) Carbodiimide-mediated cross-linking of RNA to nylon membranes improves the detection of siRNA, miRNA and piRNA by northern blot. *Nucleic Acids Res.*, **35**, e60.
37. Gustafson, A.M., Allen, E., Givan, S., Smith, D., Carrington, J.C. and Kasschau, K.D. (2005) ASRP: the Arabidopsis Small RNA Project Database. *Nucleic Acids Res.*, **33**, D637–D640.
38. Pall, G.S. and Hamilton, A.J. (2008) Improved northern blot method for enhanced detection of small RNA. *Nat. Protoc.*, **3**, 1077–1084.
39. Ascencio-Ibanez, J.T., Sozzani, R., Lee, T.J., Chu, T.M., Wolfinger, R.D., Cella, R. and Hanley-Bowdoin, L. (2008) Global analysis of Arabidopsis gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. *Plant Physiol.*, **148**, 436–454.
40. Chen, F., D'Auria, J.C., Tholl, D., Ross, J.R., Gershenzon, J., Noel, J.P. and Pichersky, E. (2003) An Arabidopsis thaliana gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. *Plant J.*, **36**, 577–588.
41. Martinez, C., Pons, E., Prats, G. and Leon, J. (2004) Salicylic acid regulates flowering time and links defence responses and reproductive development. *Plant J.*, **37**, 209–217.
42. Jung, J.H., Seo, Y.H., Seo, P.J., Reyes, J.L., Yun, J., Chua, N.H. and Park, C.M. (2007) The GIGANTEA-regulated microRNA172 mediates photoperiodic flowering independent of CONSTANS in Arabidopsis. *Plant Cell*, **19**, 2736–2748.
43. Mathieu, J., Yant, L.J., Murdter, F., Kuttner, F. and Schmid, M. (2009) Repression of flowering by the miR172 target SMZ. *PLoS Biol.*, **7**, e1000148.
44. Schwab, R., Palatnik, J.F., Riester, M., Schommer, C., Schmid, M. and Weigel, D. (2005) Specific effects of microRNAs on the plant transcriptome. *Dev. Cell*, **8**, 517–527.
45. Yamaguchi, A., Kobayashi, Y., Goto, K., Abe, M. and Araki, T. (2005) TWIN SISTER OF FT (TSF) acts as a floral pathway integrator redundantly with FT. *Plant Cell Physiol.*, **46**, 1175–1189.
46. Michaels, S.D., Himelblau, E., Kim, S.Y., Schomburg, F.M. and Amasino, R.M. (2004) Integration of flowering signals in winter-annual Arabidopsis. *Plant Physiol.*, **137**, 149–156.
47. Lee, H., Suh, S.S., Park, E., Cho, E., Ahn, J.H., Kim, S.G., Lee, J.S., Kwon, Y.M. and Lee, I. (2000) The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in Arabidopsis. *Genes Dev.*, **14**, 2366–2376.
48. Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F. and Coupland, G. (2000) Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. *Science*, **288**, 1613–1616.

49. Liu, H.H., Tian, X., Li, Y.J., Wu, C.A. and Zheng, C.C. (2008) Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. *RNA*, **14**, 836–843.
50. Zhao, B., Liang, R., Ge, L., Li, W., Xiao, H., Lin, H., Ruan, K. and Jin, Y. (2007) Identification of drought-induced microRNAs in rice. *Biochem. Biophys. Res. Commun.*, **354**, 585–590.
51. Chambers, C. and Shuai, B. (2009) Profiling microRNA expression in *Arabidopsis* pollen using microRNA array and real-time PCR. *BMC Plant Biol.*, **9**, 87.
52. Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y.Y., Sieburth, L. and Voinnet, O. (2008) Widespread translational inhibition by plant miRNAs and siRNAs. *Science*, **320**, 1185–1190.
53. Xie, Z., Kasschau, K.D. and Carrington, J.C. (2003) Negative feedback regulation of Dicer-Like1 in *Arabidopsis* by microRNA-guided mRNA degradation. *Curr. Biol.*, **13**, 784–789.
54. Vaucheret, H., Vazquez, F., Crete, P. and Bartel, D.P. (2004) The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev.*, **18**, 1187–1197.
55. Allen, E., Xie, Z., Gustafson, A.M. and Carrington, J.C. (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell*, **121**, 207–221.
56. Li, D., Liu, C., Shen, L., Wu, Y., Chen, H., Robertson, M., Helliwell, C.A., Ito, T., Meyerowitz, E. and Yu, H. (2008) A repressor complex governs the integration of flowering signals in *Arabidopsis*. *Dev. Cell*, **15**, 110–120.
57. Jang, S., Torti, S. and Coupland, G. (2009) Genetic and spatial interactions between FT, TSF and SVP during the early stages of floral induction in *Arabidopsis*. *Plant J.*, **60**, 614–625.
58. Lin, S.I., Chiang, S.F., Lin, W.Y., Chen, J.W., Tseng, C.Y., Wu, P.C. and Chiou, T.J. (2008) Regulatory network of microRNA399 and PHO2 by systemic signaling. *Plant Physiol.*, **147**, 732–746.
59. Jones-Rhoades, M.W. and Bartel, D.P. (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Mol. Cell*, **14**, 787–799.
60. Wu, G. and Poethig, R.S. (2006) Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target SPL3. *Development*, **133**, 3539–3547.
61. Li, W.X., Oono, Y., Zhu, J., He, X.J., Wu, J.M., Iida, K., Lu, X.Y., Cui, X., Jin, H. and Zhu, J.K. (2008) The *Arabidopsis* NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance. *Plant Cell*, **20**, 2238–2251.
62. Wang, J.W., Schwab, R., Czech, B., Mica, E. and Weigel, D. (2008) Dual effects of miR156-targeted SPL genes and CYP78A5/KLUH on plastochron length and organ size in *Arabidopsis thaliana*. *Plant Cell*, **20**, 1231–1243.
63. Gandikota, M., Birkenbihl, R.P., Hohmann, S., Cardon, G.H., Saedler, H. and Huijser, P. (2007) The miRNA156/157 recognition element in the 3' UTR of the *Arabidopsis* SBP box gene SPL3 prevents early flowering by translational inhibition in seedlings. *Plant J.*, **49**, 683–693.
64. Wu, G., Park, M.Y., Conway, S.R., Wang, J.W., Weigel, D. and Poethig, R.S. (2009) The sequential action of miR156 and miR172 regulates developmental timing in *Arabidopsis*. *Cell*, **138**, 750–759.
65. Wang, J.W., Czech, B. and Weigel, D. (2009) miR156-regulated SPL transcription factors define an endogenous flowering pathway in *Arabidopsis thaliana*. *Cell*, **138**, 738–749.
66. Wenkel, S., Turck, F., Singer, K., Gissot, L., Le Gourrierec, J., Samach, A. and Coupland, G. (2006) CONSTANS and the CCA1 T box binding complex share a functionally important domain and interact to regulate flowering of *Arabidopsis*. *Plant Cell*, **18**, 2971–2984.
67. Kumimoto, R.W., Adam, L., Hymus, G.J., Repetti, P.P., Reuber, T.L., Marion, C.M., Hempel, F.D. and Ratcliffe, O.J. (2008) The Nuclear Factor Y subunits NF-YB2 and NF-YB3 play additive roles in the promotion of flowering by inductive long-day photoperiods in *Arabidopsis*. *Planta*, **228**, 709–723.
68. Sunkar, R., Kapoor, A. and Zhu, J.K. (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by downregulation of miR398 and important for oxidative stress tolerance. *Plant Cell*, **18**, 2051–2065.
69. Abdel-Ghany, S.E. and Pilon, M. (2008) MicroRNA-mediated systemic down-regulation of copper protein expression in response to low copper availability in *Arabidopsis*. *J. Biol. Chem.*, **283**, 15932–15945.
70. Pant, B.D., Buhtz, A., Kehr, J. and Scheible, W.R. (2008) MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. *Plant J.*, **53**, 731–738.
71. Zhou, X., Wang, G. and Zhang, W. (2007) UV-B responsive microRNA genes in *Arabidopsis thaliana*. *Mol. Syst. Biol.*, **3**, 103.