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

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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...
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 μmol m⁻² 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 T3 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 × SSPE, 1% SDS and 0.2% BSA at 65°C for 10 min. Three successive washings were performed by using a washing solution (6 × SSPEt, 3 × SSPEt and 0.5 × 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 ~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 [zeitbeger 8 (ZT8) and ZT10]. Total RNA (10 µg) was loaded onto 17% denaturing polyacrylamide gel containing 7 M 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 3′ end were labeled at the 5′ end with [γ-32P] 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 × SSC, 0.2% SDS) at 50°C for 5, 20 and 20 min. A second washing (1 × 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 (A260/A280 > 1.8 and A260/A320 > 2.0) were used. Total RNA (1 µg) 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...
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.

**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                  | miR156a, g                                | 1.56                              |
|             | miR176g                  |                                             | 1.47                              |
|             | miR169d                  | miR169b                                    | 1.46                              |
|             | miR398a, b               | miR398b                                    | 1.41                              |
|             | miR398d                  | miR398e                                    | 1.37                              |
|             | miR398f                  | miR398i                                    | 1.36                              |
|             | miR398j                  | miR398k                                    | 1.35                              |
| 23°C        | miR398a                  |                                             | 1.29                              |
|             | miR398b                  |                                             | 1.28                              |
|             | miR398c                  |                                             | 1.20                              |

**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; miR161a2; 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

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; miR172a, c; miR395a; miR398a, b; miR399a, d, e) 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

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.

Expression analysis of 120 unique Arabidopsis miRNA loci by miRNA northern

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
miR169d 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
23°C miR398a 1.97
miR398b c 1.88
miR163a 1.61
miR172d 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
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°C 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°C 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 At1g506510 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 Cupper/Zinc Superoxide Dismutase 2 (CSD2) and At3g15640 was down-regulated at 23°C. In the case of miR399, the

| 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, SCHNARZAPFEN (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 (SOC1) (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 AP1, 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.
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 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

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**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  | ○             | ○            | ○       | ○            | ○                               |
| miR339  | ○             | ○            | ○       | ○            | ○                               |
| miR340  | ○             | ○            | ○       | ○            | ○                               |
| miR393  | ○             | ○            | ○       | ○            | ○                               |
| miR394  | ○             | ○            | ○       | ○            | ○                               |
| miR395  | ○             | ○            | ○       | ○            | ○                               |
| miR396  | ○             | ○            | ○       | ○            | ○                               |
| miR397  | ○             | ○            | ○       | ○            | ○                               |
| miR398  | ○             | ○            | ○       | ○            | ○                               |
| miR399  | ○             | ○            | ○       | ○            | ○                               |
| miR400  | ○             | ○            | ○       | ○            | ○                               |
| miR401  | ○             | ○            | ○       | ○            | ○                               |
| miR408  | ○             | ○            | ○       | ○            | ○                               |
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 (Figures 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 TOEI 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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