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Molecules and Cells

The Role of the miR399-*PHO2* Module in the Regulation of Flowering Time in Response to Different Ambient Temperatures in *Arabidopsis thaliana*

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A moderate change in ambient temperature significantly affects plant physiology including flowering time. MiR399 and its target gene *PHOSPHATE 2* (*PHO2*) are known to play a role in the maintenance of phosphate homeostasis. However, the regulation of flowering time by the miR399-*PHO2* module has not been investigated. As we have previously identified miR399 as an ambient temperature-responsive miRNA, we further investigated whether a change in expression of the miR399-*PHO2* module affects flowering time in response to ambient temperature changes. Here, we showed that miR399b-overexpressing plants and a loss-of-function allele of *PHO2* (*pho2*) exhibited an early flowering phenotype only at normal temperature (23°C). Interestingly, their flowering time at lower temperature (16ºC) was similar to that of wild-type plants, suggesting that alteration in flowering time by miR399 and its target *PHO2* was seen only at normal temperature (23°C). Flowering time ratio (16°C/23°C) revealed that miR399b-overexpressing plants and *pho2* mutants showed increased sensitivity to ambient temperature changes. Expression analysis indicated that expression of *TWIN SISTER OF FT* (*TSF*) was increased in miR399b-overexpressing plants and *pho2* mutants at 23°C, suggesting that their early flowering phenotype is associated with *TSF* upregulation. Taken together, our results suggest that miR399, an ambient temperature-responsive miRNA, plays a role in ambient temperature-responsive flowering in *Arabidopsis*.

INTRODUCTION

The initiation of flowering in *Arabidopsis* is affected by various environmental stimuli (Simpson and Dean, 2000), among which temperature plays an important role. Because plants are sessile organisms, they are continuously exposed to modest temperature changes and thus adjust their growth and development in response to moderate changes in ambient temperature. However, not much is known about how ambient temperature is sensed and triggers physiological response in plants. Recently, H2A.Z-containing nucleosomes mediate ambient temperature responses in plants (Kumar and Wigge, 2010). *SHORT VEGETATIVE PHASE* (*SVP*) was shown to control flowering time responsive to ambient temperature changes via direct binding to the *FLOWERING LOCUS T* (*FT*) locus (Lee et al., 2007). Moreover, it was also suggested that *SVP* is involved in small RNA-mediated flowering in response to ambient temperature changes (Lee et al., 2010). The integration of signals that promote or inhibit floral development in response to ambient temperature changes ultimately converge in the regulation of a few floral integrator genes including *FT* and *TWIN SISTER OF FT* (*TSF*) (Kardailsky et al., 1999; Kobayashi et al., 1999). Although some genetic evidence on the ambient temperature-responsive flowering has been reported, obviously it is still an early stage and more data should be accumulated to better interpret ambient temperature signaling.

MicroRNAs (miRNA) are small non-coding RNAs (21-22 nucleotides) that have been implicated in various plant functions, including development, phase transitions, and responses to environmental stress (Lee et al., 2010; Palatnik et al., 2003; Wang et al., 2009). The mature miRNA associates with an RNA-induced silencing complex (RISC) and guides it to the target mRNA, resulting in the inhibition of the expression of the target gene. The association between miRNA and plant development is seen in the function of miR156 and miR172, which target SQUAMOSA PROMOTER BINDING PROTEIN (SBP)-box and AP2-like family genes, respectively, to control the expression of floral integrator genes and modulate flowering time and phase transitions (Wang et al., 2009; Wu et al., 2009). Constitutive expression of miR156 and miR172 resulted in delayed and accelerated flowering times, respectively, whereas their target mimicry lines show an opposite phenotype (Franco-Zorrilla et al., 2007; Todesco et al., 2010).

MiR399, which is generated from 6 loci (miR399a, b, c, d, e, and f) in the *Arabidopsis* genome, is known to play an important role in the maintenance of Pi homeostasis (Bari et al., 2006; Chiou et al., 2006; Pant et al., 2008). Overexpression of MiR399

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results in overaccumulation of Pi (Chiou et al., 2006). *PHO2* was identified as a target gene of miR399 (Aung et al., 2006; Bari et al., 2006) based on the reduction of *PHO2* expression levels via miR399-mediated cleavage (Lin et al., 2008). The control of Pi homeostasis by miR399 is therefore mediated by the regulation of *PHO2* expression. *PHO2* encodes a ubiquitin-conjugating E2 enzyme, which is a component in the ubiquitin-dependent protein degradation pathway (Sunkar and Zhu, 2004), suggesting that protein degradation is important in Pi homeostasis. Recently, miR399 was identified as an ambient temperature-responsive miRNA (Lee et al., 2010). Mature miR399 is more abundant in plants grown at 23°C than at 16°C. The expression levels of *PHO2* are negatively correlated with miR399 expression at different temperatures, suggesting that miR399 is involved in the response to ambient temperature changes in plants. However, the role of miR399 and its target gene *PHO2* in the regulation of ambient temperature-responsive flowering time is not known.

In the present study, we studied the roles of miR399 and *PHO2* in the regulation of ambient temperature-responsive flowering time. We analyzed p35S:miR399b plants and a loss-of-function allele of *PHO2* (*pho2*). Both p35S:miR399b plants and *pho2* mutants showed early flowering only at normal temperature, thus exhibiting increased sensitivity to ambient temperature changes. *TSF* transcript levels were increased in both p35S:miR399b and *pho2* plants, which may explain their early flowering phenotype. Collectively, the present results suggest that the miR399-*PHO2* module plays a role in the regulation of flowering time in response to ambient temperature changes in plants.

MATERIALS AND METHODS

Plant materials and measurement of flowering time

Wild-type and transgenic *Arabidopsis* plants were grown on MS medium or soil at 23°C or 16°C under long day (16 h light/8 h dark) conditions at a light intensity of 120 μmol m^{-2} s^{-1}. Construction of the p35S:miR399b was described previously (Chiou et al., 2006). Flowering time was determined either by counting the total number of leaves (rosette + cauline) on the main shoot of plants grown in soil or by measuring the number of days elapsed between the time of germination and the time of bolting. Flowering time was determined by scoring at least 10 plants.

Small RNA Northern hybridization

Plants were harvested at zeitgeber time (ZT) 8. Total RNA was extracted with Plant TRIzol® Reagent (Invitrogen) from 8-day-old whole seedlings grown at 23°C or 16°C under LD conditions. For small RNA Northern blots, 10 μg of total RNA was separated on a denaturing 17% polyacrylamide gel (8 M urea) in TBE buffer and transferred to an N^+ Hybond membrane (Amersham). Hybridization was carried out at 42°C using PerfectHyb™ Plus hybridization buffer (Sigma). DNA oligonucleotide probes specific to miR399b were end-labeled with γ^{32}P-ATP using Optikinase™ (USB). The bands' intensities were quantified using Image Gauge. Expression was normalized against *U6* RNA (Yoo et al., 2011).

Gel-based RT-PCR

The reverse transcriptase-mediated PCR (RT-PCR) procedure has been described previously (Yoo et al., 2005). Total RNA was isolated from whole seedlings using Trizol® reagent (Invitrogen), according to the manufacturer's instructions. The cDNA was synthesized from 1 μg total RNA treated with DNaseI (New England Biolab). PCR cycle numbers of each gene were determined as an amplicon exponentially amplified by PCR. Resulting amplicons were separated using 1.2% agarose gel electrophoresis. *UBQ10* (At4G05320) was used as an internal positive control (Lee et al., 2010).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

The cDNA was synthesized from extracted total RNA using oligo dT primers and the First Strand cDNA Synthesis Kit for RT-PCR (Roche) and analyzed by real-time (qRT-PCR). Quantitative RT-PCR analysis was carried out according to the 'Eleven Golden Rules for Quantitative RT-PCR' (Udvardi et al., 2008). The qRT-PCR reaction was performed in a 384-well plate with a LightCycler 480 Real Time PCR system (Roche) using the KAPA SYBR® FAST qPCR Kit (Kapa Biosystems) (Li et al., 2010). LingRegPCR was used for calculating threshold cycle (C_t) and PCR efficiency of the primers used (Ramakers et al., 2003). Relative expression of the transcripts was calculated using PCR efficiency and C_t value according to the instruction of geNorm (Vandesompele et al., 2002). In order to quantify more precisely, we used two genes (At3G01150 and At4G26410) that were identified as stably expressed genes at 23°C and 16°C for reference genes during quantification (Hong et al., 2010), instead of conventional housekeeping genes. All qRT-PCR experiments were carried out in biological duplicates with technical triplicates for each. The oligonucleotide sequences of the hybridization probes and PCR primers used in this study are presented as Supplementary Table S1.

RESULTS

MiR399b overexpression caused ambient temperature-sensitive flowering

To elucidate the role of miR399 in the regulation of flowering time in response to different ambient temperatures, we analyzed the phenotype of transgenic plants overexpressing miR399b (p35S:miR399b). Before we measured flowering time of p35S:miR399b plants, we first confirmed the overproduction of miR399b in transgenic plants at 23°C and 16°C (Fig. 1A). Small RNA hybridization analysis indicated that mature miR399b was highly expressed at both temperatures (8.0-fold increase at 23°C and 7.2-fold increase at 16°C) in transgenic plants grown in normal nutrient media under Pi-sufficient conditions. This indicated that the transgenic plants we used could be used to test the effect of miR399 activity on flowering time. In addition, we found that in wild-type (WT) plants expression levels of miR399b at 23°C was higher than at 16°C, which indicated that upregulation of miR399 at 23°C was reproducible (Lee et al., 2010).

Flowering time measurement showed that transgenic p35S:miR399b plants grown at 23°C under LD conditions flowered with 11 leaves (WT plants = 14.5 leaves), showing that p35S:miR399b plants were slightly early flowering (Figs. 1B and 1C). However, the leaf number of p35S:miR399b plants grown at 16°C was similar to that of WT plants (24.6 leaves versus 24.4 leaves). This observation indicated that p35S:miR399b plants were early flowering only at 23°C. We also measured a flowering time ratio (defined as the proportion of the total number of leaves at 16°C/23°C) to determine the ambient temperature sensitivity. We used this ratio as an indicator of ambient temperature-responsive flowering. In miR399b-overexpressing plants, flowering time ratio was 2.24, which was greater than that of WT plants (1.68). This indicated that in p35S:miR399b plants flowering time variation in response to ambient temperature changes was greater than in WT plants,

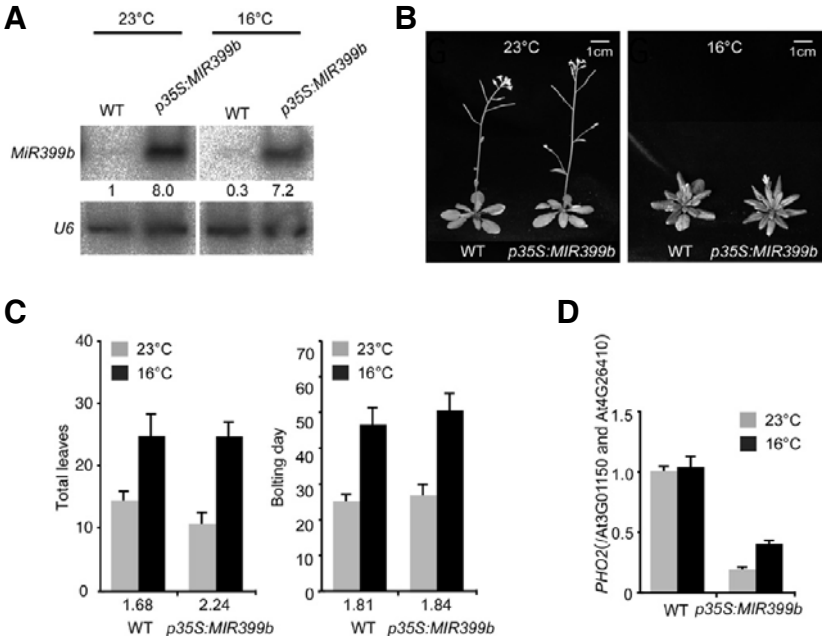

Fig. 1. Overexpression of miR399 caused an early flowering phenotype only at normal temperature. (A) Small RNA northern blot analysis of mature miR399b in 8-day-old p35S:miR399b plants grown at 23°C and at 16°C in Pi-sufficient MS medium. U6 RNA was used to show an equal amount of loading. Numbers below each blot indicate fold change relative to the miR399 level in WT plants grown at 23°C. (B, C) Phenotype and flowering time presented as total leaf number (rosette and cauline) and bolting day of p35S:miR399b plants grown at 23°C and 16°C under LD conditions. The vertical T-bars indicate standard deviation. Numbers above each genotype indicate leaf number ratio (16°C/23°C) or bolting-days ratio (16°C/23°C). (D) Quantitative RT-PCR analysis of *PHO2* transcript levels in 8-day-old p35S:miR399b plants grown at 23°C and 16°C in Pi-sufficient MS medium. *PHO2* expression levels were normalized against the expression levels of At3G01150 and At4G26410 and *PHO2* expression level in wild-type plants was arbitrarily set to 1.0.

revealing that miR399b overexpression caused ambient temperature-sensitive flowering. However, p35S:miR399b plants bolted almost at the same time as WT plants. The bolting time ratio, which is the number of days elapsed between the time of germination and the time of bolting, was similar in miR399b-overexpressing plants and in WT plants (1.84 versus 1.81). Thus, comparison of leaf numbers and bolting days indicated that the plastochron length of miR399b-overexpressing plants was decreased only at 23°C, whereas the plastochron length of p35S:miR399b plants was not altered at 16°C. These results demonstrated that miR399b overexpression affects ambient temperature-responsive flowering.

To assess the effect of miR399b overexpression on *PHO2* expression, we analyzed the transcript levels of *PHO2* in p35S:miR399b plants at both temperatures. For this experiment, we used a set of PCR primers that amplify a region containing miR399's target sites in the 5′ UTR of *PHO2*. qRT-PCR analysis showed that *PHO2* transcript levels were decreased in p35S:miR399b plants at both temperatures (Fig. 1D), indicating that miR399 overexpression significantly reduced *PHO2* expression.

Because miR399b-overexpressing plants showed an early flowering phenotype, the flowering time gene affected by miR399 overexpression was investigated. The expression levels of flowering time genes were analyzed in 8-day-old whole seedlings of WT and p35S:miR399b plants grown under LD conditions in Pi-sufficient MS medium at 23°C and 16°C. RT-PCR analysis showed that *TSF* expression was slightly upregulated in p35S:miR399b plants (Fig. 2A). Among the flowering time genes analyzed in this study, the expression of *FVE*, *SVP*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *FLOWERING LOCUS M* (*FLM*), and *FLOWERING LOCUS C* (*FLC*) was not obviously affected by miR399 overexpression (Fig. 2A) (Ausin et al., 2004; Jang et al., 2009; Moon et al., 2003; Scortecci et al., 2003; Sheldon et al., 2000). To further confirm the upregulation of *TSF* expression, a quantitative assessment of *TSF* expression levels was performed by qRT-PCR. We found that *TSF* expression was increased by 1.67-fold in p35S:miR399b plants (Fig. 2B), consistent with the RT-PCR results. These results suggested that the weak early flowering phenotype of p35S:miR399b plants was associated with the increased *TSF* expression at 23°C. Considering that *TSF* is a strong floral promoter (Yamaguchi et al., 2005), the upregulation of *TSF* explained the early flowering phenotype of miR399-overexpressing plants.

Loss-of-*PHO2* function caused an early flowering phenotype via increased *TSF* transcript level

As we observed an ambient temperature-sensitive flowering phenotype in p35S:miR399b plants, we analyzed a loss-of-function allele of *PHO2* to test whether a similar flowering phenotype was obtained from the mutant. We used *pho2* mutants, in which a point mutation in the sixth exon (from G^{2539} to A, relative to the translational start codon) causing an early termination at the 671 amino acid (Fig. 3A). The site of the early termination was at the beginning of the UBC domain and thus the mutation therefore caused the loss of the ubiquitin-conjugating activity of UBC24 in *pho2* mutants (Aung et al., 2006). We first confirmed the down-regulation of *PHO2* in *pho2* mutants by qRT-PCR. The qRT-PCR analysis showed an approximately 6.25-fold decrease in *PHO2* transcript levels (Fig. 3B). Thus, it seemed that *pho2* mutants produced truncated (possibly non-functional) PHO2 protein with low abundance.

Flowering time measurement showed that the *pho2* mutants grown at 23°C under LD conditions showed an early flowering phenotype (9.3 leaves) in comparison to WT plants (WT plants = 14.3 leaves) (Figs. 3C and 3D). However, the flowering time in *pho2* mutants at 16°C was not significantly different from that of WT plants (23.4 leaves versus 25.1 leaves). This result showed that early flowering was observed only at 23°C. We then measured a flowering time ratio to determine how ambient temperature-responsive flowering was altered. The flowering time ratio of *pho2* mutants was 2.5 (WT plants = 1.76). This indicated that in *pho2* mutants flowering time variation in response to ambient temperature changes was greater than in WT plants, indicating that loss-of-function of *PHO2* led to ambient

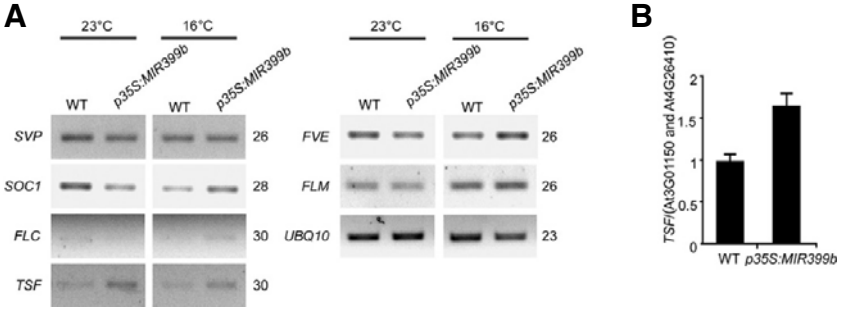

Fig. 2. Upregulation of *TSF* was observed in miR399-overexpressing plants at 23°C. (A) Expression of flowering time genes in 8-day-old p35S:miR399b plants grown at 23°C and 16°C under LD conditions analyzed by RT-PCR. *UBQ10* was used as an internal control. PCR cycle numbers are indicated on the right of each gel. (B) Relative expression level of *TSF* in 8-day-old p35S:miR399b plants grown at 23°C under LD conditions determined via qRT-PCR. The *TSF* expression level was normalized against the expression levels of At3G01150 and At4G26410. *TSF* expression level in wild-type plants at 23°C was arbitrarily set to 1.0.

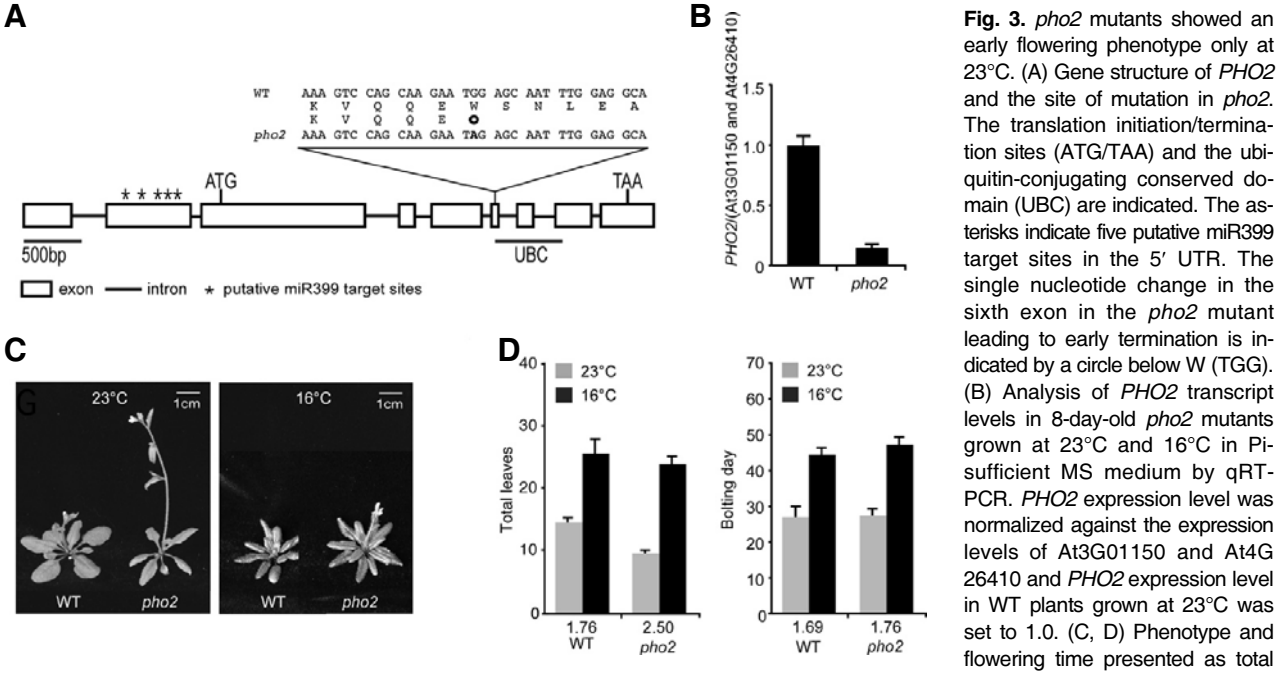

Fig. 3. *pho2* mutants showed an early flowering phenotype only at 23°C. (A) Gene structure of *PHO2* and the site of mutation in *pho2*. The translation initiation/termination sites (ATG/TAA) and the ubiquitin-conjugating conserved domain (UBC) are indicated. The asterisks indicate five putative miR399 target sites in the 5′ UTR. The single nucleotide change in the sixth exon in the *pho2* mutant leading to early termination is indicated by a circle below W (TGG). (B) Analysis of *PHO2* transcript levels in 8-day-old *pho2* mutants grown at 23°C and 16°C in Pi-sufficient MS medium by qRT-PCR. *PHO2* expression level was normalized against the expression levels of At3G01150 and At4G 26410 and *PHO2* expression level in WT plants grown at 23°C was set to 1.0. (C, D) Phenotype and flowering time presented as total leaf number and bolting day of *pho2* mutants grown at 23°C and 16°C under LD conditions. Numbers above each genotype indicate leaf number ratio (16°C/23°C) or bolting-days ratio (16°C/23°C). The vertical T-bars indicate standard deviation.

temperature-sensitive flowering, as seen in p35S:miR399b plants (Figs. 1C and 3D). However, the bolting time ratio of *pho2* mutants was similar to that of WT plants (1.76 versus 1.69), revealing that these mutants also displayed a decreased plastochron length only at 23°C. The decreased plastochron length of *pho2* mutants at 23°C was consistent with the phenotype of p35S:miR399b plants (Fig. 1C).

As *pho2* mutants showed an early flowering phenotype, we investigated the expression levels of flowering time genes in *pho2* mutants under LD conditions to determine which flowering time gene's expression was altered. RT-PCR analysis indicated that *TSF* expression was significantly elevated at 23°C in *pho2* mutants (Fig. 4A). Among the flowering time genes analyzed, the transcript levels of *FCA*, *FVE*, *SVP*, *SOC1*, *FRUIT-FULL* (*FUL*), *APETALA 1* (*AP1*), *FLM*, and *FLC* remained unaltered in *pho2* mutants (Ausin et al., 2004; Jang et al., 2009; Macknight et al., 1997; Moon et al., 2003; Scortecci et al., 2003; Sheldon et al., 2000; Wang et al., 2009). To further confirm the upregulation of *TSF* expression in *pho2* mutants, a quantitative assessment of *TSF* expression levels was performed via qRT-PCR. We found that *TSF* expression was increased by 4.5-fold in *pho2* mutants at 23°C (Fig. 4B), consistent with the RT-PCR results. Interestingly, *TSF* expression was not upregulated in *pho2* mutants grown at 16°C. Since *pho2* mutants showed an early flowering only at 23°C, these results suggested that an increase in *TSF* levels downstream of *PHO2* is responsible for the ambient temperature-responsive flowering of *pho2* mutants.

DISCUSSION

In this study, we analyzed the flowering time of miR399-overexpressing plants and a loss-of-function allele of *PHO2,* which is a target of miR399. We identified that alteration of miR399 and *PHO2* activity caused early flowering only at normal temperature, suggesting that the miR399-*PHO2* module regulates ambient temperature-responsive flowering in *Arabidopsis*.

Previous studies have described the close relationship between plant miRNAs and flowering time regulation. *GI*-regulated miR172 was shown to function in a genetic pathway that controls photoperiodic flowering by inducing *FT* in a *CONSTANS* (*CO*)-independent manner (Jung et al., 2007). A mutant, in which *TARGET OF EAT 1* (*TOE1*), the target gene of

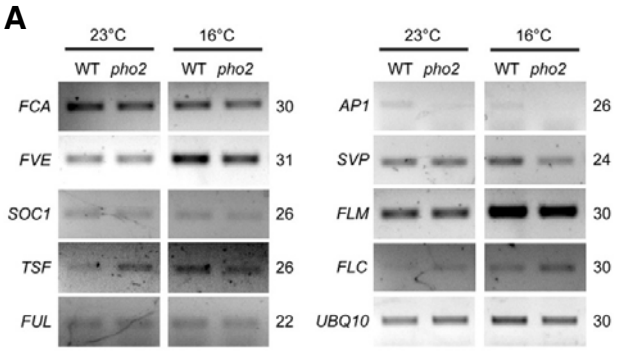

Fig. 4. Upregulation of *TSF* was observed in *pho2* mutants at 23°C. (A) Expression of flowering time genes in 8-day-old *pho2* mutants grown at 23°C and 16°C under LD conditions via RT-PCR. *UBQ10* was used as an internal control. PCR cycle numbers are indicated on the right of each gel. (B) Relative expression level of *TSF* in 8-day-old *pho2* mutants grown at 23°C under LD conditions via qRT-PCR. *TSF* expression level in wild-type plants at 23°C was arbitrarily set to 1.0.

miR172, is constitutively activated by the 35S enhancer, responded to vernalization and gibberellic acid treatment but not to day length changes. *FT* expression levels in this mutant were significantly reduced without affecting the expression of other flowering time genes. MiR172-overexpressing plants showed an early flowering phenotype under both LD and SD conditions, even in the absence of functional *CO*. In the present study, the overexpression of miR399b and a loss-of-function allele of *PHO2* caused early flowering by increasing *TSF* expression. MiR399-mediated *PHO2* cleavage may regulate photoperiodic flowering in a *CO*-independent manner based on our preliminary qRT-PCR results revealing that *CO* expression was not altered in these mutants (data not shown). Further analysis on miR399-overexpressing plants and *pho2* mutants under both LD and SD conditions is required to precisely define the role of miR399-*PHO2* in the photoperiod pathway.

The regulation of plastochron length by miRNA and its target genes has recently been reported. Down-regulation of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9* (*SPL9*) expression by miR156 overexpression resulted in reduced plastochron length and increased leaf numbers in *Arabidopsis*. Conversely, increased levels of *SPL9* as a result of decreased miR156 expression caused an increase in plastochron length (Wang et al., 2008). Our study reveals that miR399-mediated *PHO2* downregulation has an opposite effect against miR156-*SPL* in the regulation of plastochron length. Both p35S:miR399b plants and *pho2* mutants showed a reduced number of rosette leaves but they bolted at the same time as WT plants at both 23°C and 16°C (Figs. 1C and 3D), suggesting that the miR399-targeted *PHO2* module may also play a role in the regulation of plastochron length.

Devaiah and his colleagues demonstrated the relationship between phosphate starvation responses and gibberellic acid biosynthesis through studies with *MYB62*-overexpressing plants (Devaiah et al., 2009). *MYB62* is an R2R3-type transcriptional factor that is induced in response to Pi deficiency. Overexpression of *MY-B62* resulted in a GA-deficient phenotype characterized by delayed germination, bolting of the inflorescence stalk and rosette leaf initiation, and an increased number of rosette leaves. *MYB62*-overexpressing plants suppressed the transcript level of *SOC1* and *SUPERMAN* (*SUP*), the transcription factors associated with floral homeotic genes. In the present study, 35S:miR399b plants and *pho2* mutants showed early flowering via increased *TSF* expression. Overexpression of miR399 and loss-of-function of *PHO2* were reported to induce overaccumulation of Pi in the shoot and result in Pi toxicity symptoms such as necrosis or chlorosis on the margins of mature leaves. Pi overaccumulation was found to be the result of increased Pi uptake (Aung et al., 2006; Chiou et al., 2006). Taken together, our results suggest that miR399 and its target gene *PHO2* not only affect Pi homeostasis but also impact ambient temperature-responsive flowering. Although genetic evidence that the miR399-*PHO2* module regulates flowering time was presented, the possibility that the alteration of flowering time seen in p35S:miR399b and *pho2* plants was an indirect effect of Pi toxicity cannot be ruled out. Further detailed study is required to provide further evidence connecting the miR399-*PHO2* module to flowering time regulation. A possible approach would be the measurement of flowering time and expression levels of floral integrator genes in miR399-overexpressing plants and *pho2* mutants grown under high and low Pi conditions.

In conclusion, our study suggests that miR399 and its target gene *PHO2,* the genes that were known to function in the maintenance of Pi homeostasis in plants, plays a role in regulating flowering time in response to ambient temperature changes. We believe that this information will help improve the understanding of the molecular mechanism underlying the regulation of flowering time by small RNAs in response to ambient temperature changes.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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