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 (16°C) 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., 2008). 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 (Bar et al., 2006; Chiu 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 p3SS: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 (2T) 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) 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 DNasel (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 (Ct) and PCR efficiency of the primers used (Ramakers et al., 2003). Relative expression of the transcripts was calculated using PCR efficiency and C 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 (p3SS:miR399b). Before we measured flowering time of p3SS: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 p3SS:miR399b plants grown at 23°C under LD conditions flowered with 11 leaves (WT plants = 14.5 leaves), showing that p3SS:miR399b plants were slightly early flowering (Figs. 1B and 1C). However, the leaf number of p3SS:miR399b plants grown at 16°C was similar to that of WT plants (24.6 leaves versus 24.4 leaves). This observation indicated that p3SS: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 p3SS:miR399b plants flowering time variation in response to ambient temperature changes was greater than in WT plants,