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Characterization and functional analysis of microRNA399 in *Cunninghamia lanceolata*

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Abstract

The miR399 is a conserved microRNA (miRNA) family, and it has been characterized as an essential regulator of phosphorus transport in plants. However, the biological function of miR399 in *Cunninghamia lanceolata* is still largely unclear. In this study, the comparison of mature miR399 sequence revealed a high similarity between *Arabidopsis thaliana* and *C. lanceolate*, and the pre-miR399 was capable of forming a typical stem-loop hairpin structure. A gene *PHOSPHATE 2* (*PHO2*) was identified as a target of *cln*-miR399 using 5' rapid amplification of cDNA ends. Furthermore, the relationship between *cln*-miR399 and *PHO2* was further confirmed through a transient co-expression of both genes in *Nicotiana benthamiana*. To examine the function of miR399 in *Arabidopsis*, miR399-overexpressing transgenic *Arabidopsis thaliana* was acquired using *Agrobacterium*-mediated approach. Real-time PCR showed that the amount of *cln*-MIR399 transcripts was higher in miR399-overexpressing plants than in wild-type plants, which was accompanied with down-regulation of expression of its target gene *AtPHO2*. The P content was 1.40 to 1.56-fold higher in the leaves of three transgenic lines than in wild type plants. However, the P content in the roots of the three transgenic lines was 24.5 - 37.2 % less than that in wild type plants. Moreover, the transcriptions of three phosphate transporter genes (*PHT1*, *PHT2*, and *PHT3*) were up-regulated in roots of miR399-overexpressing *Arabidopsis* plants. Interestingly, the transgenic lines exhibited retarded growth under normal P conditions compared with the wild type. Our findings demonstrate that *cln*-miR399 may play crucial roles in P transport and plant growth *via* regulation of its target gene *PHO2*.

Additional key words: *Arabidopsis thaliana*, *Nicotiana benthamiana, PHO2*, phosphate transporters, RLM-RACE.

Introduction

MicroRNAs (miRNAs) represent a class of 20 - 24 nucleotide (nt) non-coding small RNAs, which can regulate target mRNAs *via* directing cleavage or translational repression (Voinnet 2009, Cuperus *et al*. 2011). An increasing number of evidence indicates that miRNAs play critical roles in leaf morphogenesis, phase transitions, nutrient homeostasis, and various biotic or abiotic stress responses (Qiu *et al*. 2013, 2016, Hu *et al*. 2015, Hai *et al*. 2018).

The microRNA399 (miR399) is one of the most ancient and highly conserved microRNA families in monocots and dicots (Cuperus *et al*. 2011). Recently, miR399 has been demonstrated to target the *PHOSPHATE 2* (*PHO2*) gene, which has been confirmed by using a modified 5'-rapid amplification of cDNA ends (RACE) method. The *PHO2* encodes an ubiquitin-conjugating E2 enzyme involved in ubiquitin-mediated protein degradation. In *Arabidopsis* and rice, miR399 has been confirmed to act as an important regulator of P acquisition and P metabolism through the downregulation of *PHO2* transcription (Bari *et al*. 2006, Chiou *et al*. 2006), and rice transgenic lines with miR399 overexpression exhibit multiple nutrient starvation responses (Hu *et al*. 2015). Furthermore, the overexpression of miR399 in transgenic plants could increase the content of sugars and vitamin C, thus improving the quality of strawberry fruits (Wang *et al*. 2017). These findings suggest that miR399 acts mainly *via* down-regulating the target gene *PHO2* to modulate a wide range of metabolic and other biological processes.

Chinese fir (*Cunninghamia lanceolata* Lamb. Hook) is one of the most important coniferous evergreen tree species due to its rapid growth, and thus it serves as a global resource of wood (Shi *et al*. 2010, Wan *et al*. 2012). A genome-scale analysis of miRNA expression

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Abbreviations: Pi - inorganic phosphorus; miRNA - microRNA; nt - nucleotide; PHO2 - PHOSPHATE 2; RACE - 5' rapid amplification of cDNA ends; RLM - RNA ligase-mediated.
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profiling in *C. lanceolata* was carried out by using high-throughput sequencing, and 20 known miRNAs families including miR156, miR159, miR172, miR396, miR399, and miR408 have been identified in our previous study (Wan *et al*. 2012, Qiu *et al*. 2015). Although more and more studies have demonstrated the critical roles for miR399 and its targets *PHO2* in *Arabidopsis*, tomato, and strawberry (Bari *et al*. 2006, Gao *et al*. 2015, Wang *et al*. 2017); relatively less is known about the function of miR399 and its targets in *C. lanceolata*. To evaluation the roles of miR399 in mediating P transport and plant growth, transgenic *Arabidopsis* with *cln*-MIR399 overexpression were generated using *Agrobacterium*-mediated approach. Furthermore, the expression of the miR399 precursor, *PHO2* mRNA, as well as inorganic phosphorus (Pi) content were analyzed in wild-type and miR399-overexpressing *Arabidopsis*. Subsequently, the phenotypes of transgenic *Arabidopsis* ectopically expressing c*ln*-miR399 were characterized. These results could deliver a new insight into the function of c*ln*-miR399 and its target and could provide the basis for further functional studies of miRNA in conifers.

Materials and methods

Plants and growth conditions: *Arabidopsis thaliana* L. ecotype Columbia (Col-0) was used to obtain transgenic lines. The wild type, transgenic *Arabidopsis* plants and *Nicotiana benthamiana* Domin seedlings were grown in a greenhouse at a temperature of 22 °C, a 16-h photoperiod, an irradiance of 120 μmol m^{-2} s^{-1}, and a realtive humidity of 70%. Seeds of Chinese fir (*Cunninghamia lanceolata* Lamb. Hook) were obtained from the Fujian Academy of Forest Sciences, Fujian, China and small seedlings were grown under above mentioned conditions. The seedlings (7-d-old) of wild type and transgenic *Arabidopsis* plants showing consistent growth were transplanted into soil supplemented with a one-half Hoagland nutrient solution containing 250 μM KH_2PO_4 (the normal phosphorus concentration).

Sequence analysis: Precursor sequences of miR399 in *C. lanceolata* were examined for the secondary structure using the *Mfold* program with the default parameters (Zuker 2003). Mature miR399 sequences of *Arabidopsis* were downloaded from *miRBase release 21.0* (http://www.mirbase.org). Multiple sequence alignments of *cln*-miR399 and *Arabidopsis* miR399a-f mature sequences were performed using the *DNAMAN* software.

To define target genes of *cln*-miR399, the mature sequence of *cln*-miR399 was used as a query to search against the Chinese fir mRNA transcriptome database (59 669 sequences) using the web-based program *psRNATarget* with default parameters (Zhang 2005). The sequence of *PHO2* (a ubiquitin-conjugating E2 enzyme) was obtained from *Arabidopsis thaliana* (*TAIR v. 10*).

Rapid amplification of cDNA ends: A modified RNA ligase-mediated 5' rapid amplification of cDNA ends (RLM-RACE) was conducted to obtain cleavage transcripts based on the method described by Ding *et al*. (2016). Gene-specific primers are listed in Table 1 Suppl.

Transient co-expressions of *cln*-MIR399 and *PHO2*: The sequence of the miR399 precursor was amplified from the genomic DNA in three-month-old *C. lanceolata* seedlings with the following primer pairs: forward, 5'-GGGGATAATTACTGGGGGACTCTTC-3'; reverse, 5'-AGAACAATTGCAGGGCAACTCTCCT-3'. The amplified fragment was introduced into a binary vector (pCAMBIA2300) under the control of the cauliflower mosaic virus 35S promoter. The target gene *PHO2* was amplified from *Arabidopsis* or *C. lanceolata* with primers listed in Table 1 Suppl. and inserted into the same vector. *Agrobacterium* suspensions harboring *cln*-MIR399 and *PHO2* were infiltrated separately or coinfiltrated into the leaves of 4-week-old tobacco as previously described (Zheng *et al*. 2012). For co-expression of *cln*-MIR399 and PHO2, two kinds of *Agrobacterium* suspensions were mixed at a 1:1 ratio before infiltration of tobacco leaves.

Cloning and overexpression of cln-MIR399 in Arabidopsis: To overexpress *cln*-MIR399 in *Arabidopsis*, pre-miR399 was cloned *via* reverse transcription PCR amplification using primers 5'-GGGGATAATTACTGGGGGACTCTTC-3' and 5'-AGAACAATTGCAGGGCAACTCTCCT-3'. The amplified fragments were sequenced and then subcloned into the binary vector pCAMBIA2300 between the *Kpn*I and *Bam*HI sites to generate a 35S:MIR399 construct. The construct containing the 35S promoter was transformed into the Agrobacterium tumefaciens strain EHA105 and was then transferred into wild-type Col-0 ecotype plants using the method proposed by Bechtold and Pelletier (1998). Transgenic seeds were screened on a medium containing kanamycin and validated by PCR amplification. Subsequently, T_3 homozygous lines were used for phenotypic characterization and gene expression analysis.

Real-time quantitative PCR: Total RNA isolation, cDNA synthesis from the total RNA, and followed real-time quantitative PCR analysis were conducted according to Qiu *et al*. (2016) and Hai *et al*. (2018). For amplification, specific primers were designed for *cln*-MIR399, *AtPHO2*, *PHT1*, *PHT2*, and *PHT3* (Table 1 Suppl.). Relative expressions of different genes were normalized against an internal reference gene *Arabidopsis tubulin* using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Determination of total P content: Roots and shoots from 7-week-old wild-type plants and MIR399-overexpressing transgenic *Arabidopsis* lines were harvested and immediately frozen in liquid nitrogen. Total P content was determined according to the method proposed by Hu *et al*. (2015). Briefly, the leaves and roots were dried at 80 °C to a constant weight. Dried samples (50 mg) were digested with 13 cm^3 of concentrated HNO_3 and 2 cm^3 of 30 % H_2O_2 at 140 °C for 30 min. The digested solutions were adjusted to a volume of 25 cm^3 with de-ionized water. The metal

elements were determined using inductively coupled plasma optical emission spectrometry (*PE*, *Optima 2000 DV*, Waltham, MA, USA).

Statistics: All samples were carried out in three biological triplicates, and results were represented as means ± SEs of three replicates. For evaluation of significant differences at $\alpha = 0.05$, the Duncan's multiple range test was used.

Results

The miR399 family belongs to conserved miRNA families across diverse plant species, and *cln*-miR399 has been identified from *C. lanceolata* in our previous study based on high-throughput sequencing (Qiu *et al*. 2015). Mature sequences of *Arabidopsis* miR399 family members were derived from *miRBase release 21*. The alignment of mature sequences in *C. lanceolata* miR399 and *Arabidopsis* miR399a-f were conducted using the multiple sequence alignment method. The results show that the mature sequence of *cln*-miR399, which was 21-nt long (5'-UGCCAAAGGAGAGUUGCCCUG-3'), was the same as *Arabidopsis* mature miR399b and miR399-3p sequences (Fig. 1) indicating that the miR399 family sequence was deeply conserved. The precursor of *cln*-miR399 was amplified by PCR, and the 105 bp precursor sequence was capable of forming a stable stem-loop secondary structure (Fig. 1 Suppl.). The minimum folding free energy index of the pre-miR399 hairpin structure was 0.98, and the average A+U content of the pre-miR399 sequence was 58.1 (Table 1). As shown in Table 2, two potential target genes, *PHOSPHATE 2* (*PHO2*) (*unigene56556*) and *predicated protein* (*unigene84522*) were predicted from the *C. lanceolata* mRNA transcriptome database based on *psRNATarget* analysis.

ath-miR399a	5'	UGCCAAAGGAGAUUUGCCCUG	3'
ath-miR399d	5'	UGCCAAAGGAGAUUUGCCCCG	3'
ath-miR399e	5'	UGCCAAAGGAGAUUUGCCUCG	3'
ath-miR399f	5'	UGCCAAAGGAGAUUUGCCCGG	3'
ath-miR399b	5'	UGCCAAAGGAGAGUUGCCCUG	3'
ath-miR399c-3p	5'	UGCCAAAGGAGAGUUGCCCUG	3'
cln-miR399a	5'	UGCCAAAGGAGAGUUGCCCUG	3'

Fig. 1. Alignment of mature sequences in *Cunninghamia lanceolata* miR399 (*cln*-miR399a) and *Arabidopsis* miR399 (*ath*-miR399a-f). Mismatches are *underlined*.

To evaluate whether miR399 could mediate the cleavage of putative target mRNA in *C. lanceolata*, we detected the cleavage products of *unigene56556* mRNA in 3-month-old *C. lanceolata* seedlings using 5'-RACE. The *unigene 56556* encoding *PHO2* had a cleavage site at the 10th and 11th nucleotide of miR399 from the 5′-end (Fig. 2). The cleavage event was further verified by A*grobacterium*-mediated transient co-expression in *N. benthamiana.* The results of transient co-expression demonstrate that the amount of *PHO2* transcripts markedly decreased by *cln*-miR399 (Fig. 3). These results provide direct evidence that the *PHO2* gene is the true target of miR399 in Chinese fir.

To explore the function of *C. lanceolata* miR399, we generated transgenic *Arabidopsis* plants overexpressing *cln*-miR399 driven by the enhanced cauliflower mosaic virus 35S promoter. Three independent transgenic lines were selected for further analysis. To further determine the

Table 1. Characteristics of a miR399 precursor from Chinese fir. MFE - minimum folding free energy, MFEI - minimal folding free energy index.

miRNA	cln-miR399
Mature sequence	UGCCAAAGGAGAGUUGCCCUG
Mature miRNA length [nt]	21
Precursor sequence	GGGGAUAAUUACUGGGGGACUCUUCUUUGGCUGGAAUUAUUCAAUCUCUUAAC
	UCAUGUUAAUGUUUUGUUGCCUGCCAAAGGAGAGUUGCCCUGCAAUUGUUCU
Pre-miRNA length [nt]	105
Arm location	3'
MFE [kJ mol-1]	-43.20
A+U [%]	58.1
MFEI	0.98

Table 2. Predicted target genes for miR399 and their putative functions.

miRNA	Target genes	Score	Predicted function	GO annotation
cln-miR399	*unigene56556*	0.5	phosphate 2	phosphate homeostasis
	unigene84522	0.5	predicated protein	

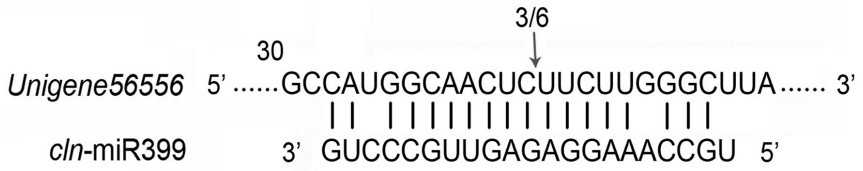

Fig. 2. The 5' rapid amplification of cDNA ends of miRNA cleavage sites: the miRNA sequence (*bottom*) and its corresponding target mRNA sequence (*top*) is shown in each alignment. The *arrow* indicates a cleavage site detected by RNA ligase-mediated 5' rapid amplification of cDNA ends, and the *number* above the *arrow* indicates the number of cleaved/total clones sequenced.

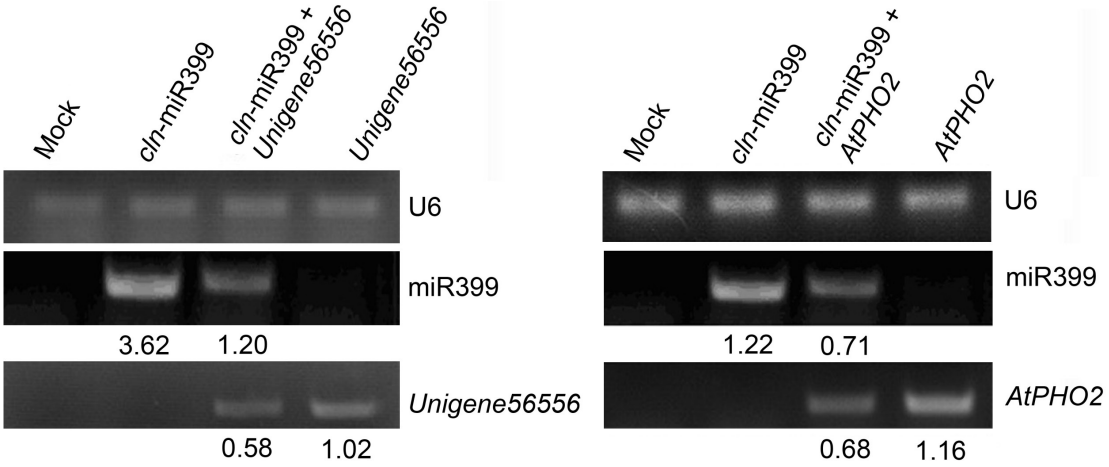

Fig. 3. Transient co-expression assay of *Cunninghamia lanceolata cln*-miR399 (*A*,*B*) and *C. lanceolata unigene56556* (*A*) and *Arabidopsis PHO2* (*B*) in *Nicotiana benthamiana* leaves. Accumulations of mRNA of *cln*-miR399, *unigene56556*, and *PHO2* are shown. Leaves transformed with an empty vector served as a loading control (Mock).

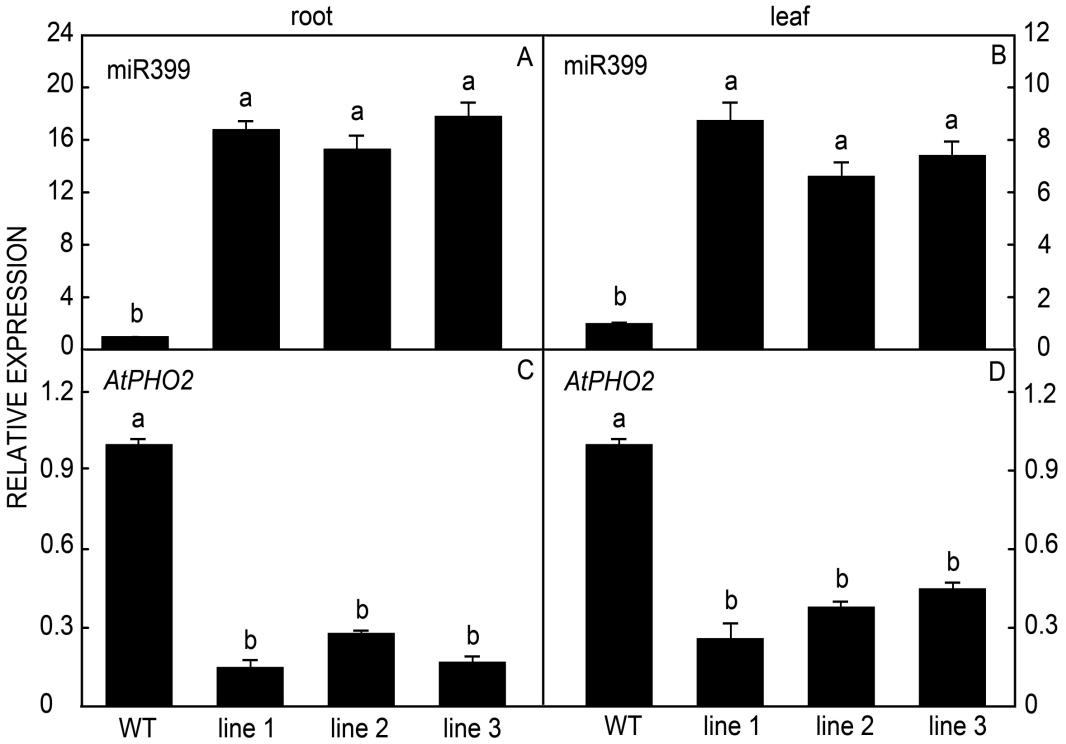

Fig. 4. Expression analysis of *cln*-MIR399 (*A*, *C*) and its target *PHO2* (*B, D*) in the roots and leaves of wild type (WT) and transgenic lines 1, 2, and 3 under normal P conditions. The expressions of miR399 and *PHO2* in the WT were set to 1. Means ± SEs, $n = 3$, different letters indicate significant differences according to the Duncan's multiple range test at $P \leq 0.05$.

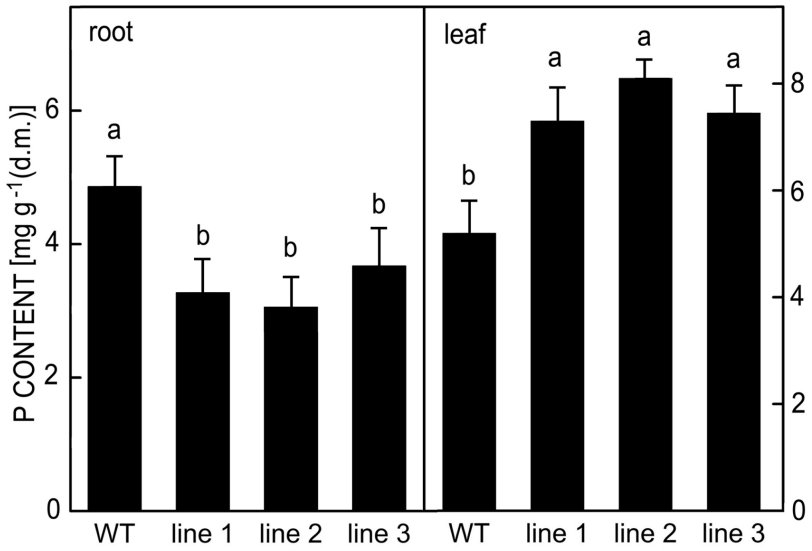

Fig. 5. Phosporus content in leaves and roots of seven-week-old wild-type and transgenic *Arabidopsis* lines grown at normal P conditions. Means ± SEs, $n = 3$; different letters indicate significant differences according to the Duncan's multiple range test at $P \leq 0.05$.

expression of *cln*-MIR399 in the three lines, total RNA was isolated from 4-week-old wild type *Arabidopsis* plants and transgenic lines and analyzed by real-time quatitative PCR. As shown in Fig. 4, the transcription of *cln*-MIR399 in the three independent transgenic lines was much higher than in the wild-type plants. Interestingly, *cln*-MIR399 was also more expressed in roots of transgenic plants than in their leaves. A high miR399 accumulation in transgenic plants suggests that *cln*-MIR399 was successfully expressed in *Arabidopsis*. By contrast, the target gene *PHO2* transcriptions were substantially lower in the roots and leaves of the three transgenic lines exhibiting an opposite trend of expression to *cln*-MIR399 suggesting that the *PHO2* mRNA abundance could be negatively regulated by miR399. To further examine a relationship between *cln*-MIR399 and *AtPHO2*, *Agrobacterium*-mediated transient co-expressions of *cln*-MIR399 and *AtPHO2* in *N. benthamiana* were established. The results of the transient co-expressions show that AtPHO2 markedly decreased by *cln*-MIR399 (Fig. 3) suggesting that miR399 could directly cleavage and degrade mRNA of *PHO2*.

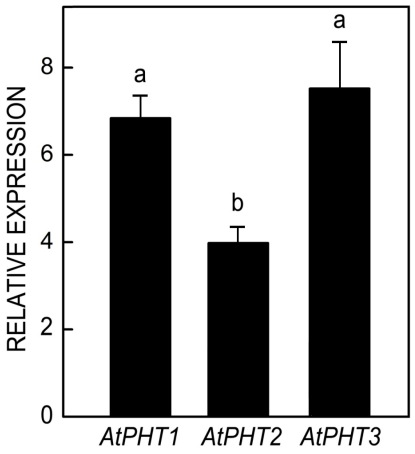

Fig. 6. Transcript abundances of three phosphate transporter genes in transgenic *Arabidopsis* lines under normal P conditions. The expression in the wild type was set to 1. Means ± SEs, $n = 3$.

To assess whether miR399 overexpression affected P transport and plant growth, we grew seven-week-old wild type *Arabidopsis* plants and miR399-overexpressing *Arabidopsis* lines at normal P conditions in soil for 5 d. After 5 d, we measured the total P content in transgenic lines and wild-type plants. The P content increased 1.40 to 1.56-fold in the leaves of the three transgenic lines compared with the wild-type plants. However, the P content in the roots of the three transgenic lines was 24.5 to 37.2 % less than that in the wild-type plants (Fig. 5).

To estimate the effect of miR399 on Pi transport and accumulation in leaves, we next used real-time quantitative PCR to analyze the expressions of the three *phosphate transporter* (*PHT*) genes in transgenic *Arabidopsis* plants and wild-type plants. As a result, relative expressions of *AtPHT1*, *AtPHT2*, and *AtPHT3* increased more in roots of transgenic lines in comparison with wild-type plants at normal P conditions (Fig. 6) indicating that they were positively regulated by miR399 and important for Pi transport and accumulation in *Arabidopsis*.

To investigate the effect of the miR399 overexpression on growth of the plants, we characterized the phenotype of the miR399-overexpressing plants. As shown in Fig. 7, two-week-old transgenic plants displayed shorter roots than wild type plants. Furthermore, compared with the wild type, seven-week-old transgenic plants exhibited a retarded growth.

Discussion

The miR399 is a conserved miRNA family existing in several plant species, and this family is predicted to target

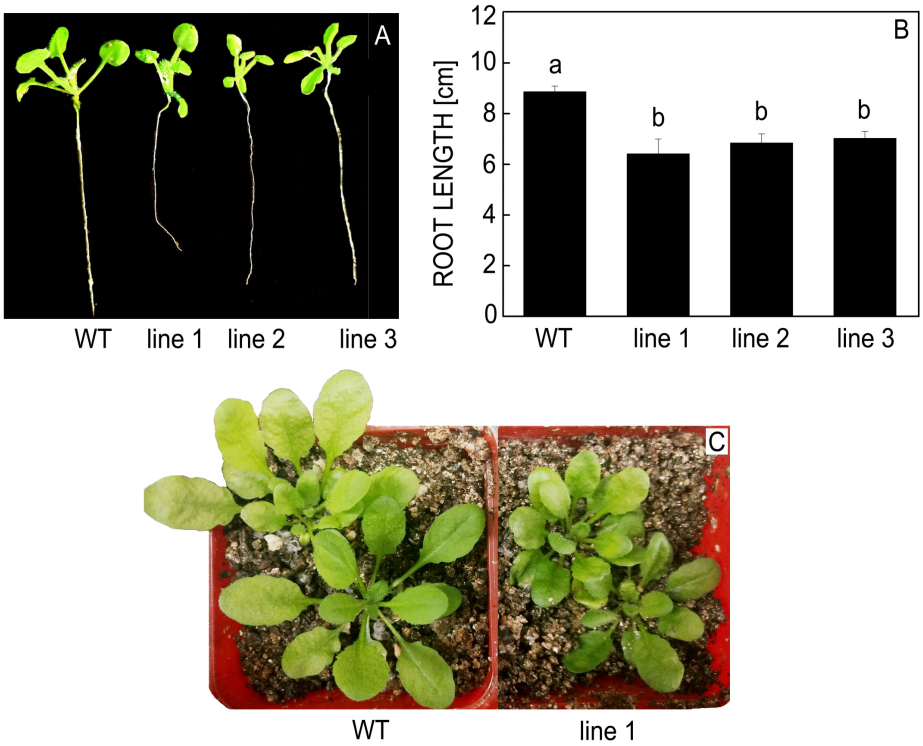

Fig. 7. Growth features of wild type and transgenic *Arabidopsis* plants grown under normal P conditions in soil determined two (*A*) and seven (*C*) weeks after seed germination. *B* - Root length of wild type (WT) and transgenic *Arabidopsis* plants. Means ± SEs, $n = 15$. Different letters indicate significant differences according to the Duncan's multiple range test at $P \leq 0.05$.

PHO2 (Lin *et al*. 2008, Hackenberg *et al*. 2013). In the present investigation, we searched for putative target genes for *cln*-miR399 by bioinformatics prediction and identified two targets, *PHO2* (*unigene 56556*) and *predicated protein* (*unigene 84522*) as a candidate target for miR399. Similar to that in *C. lanceolata*, miR399 also targets *PHO2* genes in *Arabidopsis*, barley, and strawberry (Lin *et al*. 2008, Hackenberg *et al*. 2013, Wang *et al*. 2017). To test whether *PHO2* is subjected to miRNA-mediated cleavage *in vivo*, we isolated mRNAs from three-month-old *C. lanceolata* seedlings and performed 5'-RACE method to detect the 3' cleavage products. A 5′-RACE assay is a simple and efficient method for *in vivo* assays of the cleavage sites of miRNAs on their mRNA targets. Here, *PHO2*, which encodes *PHOSPHATE 2*, was verified and characterized as the target gene of *cln*-miR399 by 5'-RACE suggesting that the target identified by bioinformatics prediction is indeed authentic, and miR399 can target and cleave the corresponding transcripts in *C. lanceolata*.

To confirm whether *Arabidopsis PHO2* transcripts could be directly cleaved by *cln*-MIR399, we investigated a relationship between *cln*-MIR399 and *AtPHO2* through transient co-expressions of both genes in tobacco leaves. Interestingly, *cln*-MIR399 expression markedly decreased when co-expressed with 35S::*cln*-MIR399. Our results are consistent with the results of Wang *et al*. (2010), who demonstrated that miR399 can cleave *PHO2* mRNA in *Arabidopsis* and tobacco leaves. Our results show that *Arabidopsis PHO2* was a target of *cln*-MIR399 and that *cln*-MIR399 had the capability to direct the cleavage of *AtPHO2 in vivo*.

To further dissect the relationship between *cln*-miR399 and its target *PHO2*, the transcriptions of miR399 and *AtPHO2* were monitored in the 35S: *cln*-MIR399 transgenic plants and the wide-type plants by real-time quantitative PCR. The results show that miR399 was successfully up-regulated in *cln*-miR399-overexpressing plants as compared with wild type plants. Interestingly, *cln*-miR399 expression in the roots of transgenic plants was much higher than in the leaves. Our results are in agreement with a study in tomato, in which miR399 is highly expressed in roots of *miR399*-overexpressing plants (Gao *et al*. 2015). Additionally, the expression analysis revealed a significant down-regulation of miR399 target genes (*AtPHO2*) in both the roots and leaves of *cln*-miR399-overexpressing plants in comparison with the wild type and exhibited a negative impact on the expression of miR399. Taken together, these results suggest that miR399 derived from a *C. lanceolata* precursor was heterologously expressed in *Arabidopsis*, and miR399 controlled a wide range of metabolic processes in *C. lanceolata* by a negative regulation of *PHO2*.

Inorganic phosphorus is one of the most significant mineral nutrients for plants as it constitutes many important biological molecules such as nucleic acids, phospholipids, and ATP. In soil solution, the concentration of Pi is very low, and consequently, P starvation often occurs. Recently, a new type of regulatory element, miR399, has been confirmed to act as a mediator in improving P uptake and translocation under P deficiency conditions in *Arabidopsis*

and rice (Chiou *et al*. 2006, Hu *et al*. 2015). The line overexpressing miR399 results in P accumulation in shoots of *Arabidopsis* (Fujii *et al*. 2005), strawberry (Wang *et al*. 2017), and tomato (Gao *et al*. 2015). These results are in agreement with our results where overexpression of *cln*-miR399 led to increased P content in the leaves of *Arabidopsis*. However, the P content in the roots of transgenic lines was lower than that in the wild type. The lower P content in the roots of transgenic *Arabidopsis* is consistent with results obtained on transgenic woodland strawberry (Wang *et al*. 2017). These results suggest that the overexpression of miR399 could enhance P transfer ability and affect P distribution from roots to shoots. We next considered whether overaccumulation of P in the leaves resulted from an increased uptake of P. Therefore, transcriptions of *AtPHT1*, *AtPHT2*, and *AtPHT3*, which are important phosphate transporters controlling P uptake and translocation, were analyzed by real-time quantitative PCR. We found that the expression of *AtPHT1*, *AtPHT2*, and *AtPHT3* more increased in the roots of transgenic lines than in the wild type at the normal P conditions. These results are in agreement with that of Chiou *et al*. (2006), who showed that the expressions of two phosphate transporter genes, *AtPHT1* and *AtPHT2* in roots of miR399-overexpressing *Arabidopsis* are higher than in a wild type. Gao *et al*. (2015) also reported that overexpressing ath-miR399d enhances the transcriptions of *phosphate transporters LePT1*, *LePT2*, *LePT4*, and *LePT5* in P-sufficient roots of transgenic tomato. Therefore, miR399 activated by down-regulating *PHO2* the expression of P transporters, such as *AtPHT1* and *AtPHT2*, and consequently enhanced P uptake and transport to shoots.

In summary, this study demonstrated that miR399 derived from a *C. lanceolata* precursor together with its target gene *PHO2* were confirmed to be critical in regulating P transport and plant growth. The precise mechanisms how *cln*-miR399 regulates the P transport and plant growth should be further elucidated.

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