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. lanceolata, 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 miRNAs 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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Submitted 23 April 2019, last revision 18 October 2019, accepted 8 January 2020.

Abbreviations: Pi - inorganic phosphorus; miRNA - microRNA; nt - nucleotide; PHO2 - PHOSPHATE 2; RACE - 5’ rapid amplification of cDNA ends; RLM - RNA ligase-mediated.

Acknowledgements: This work was supported by the National Natural Science Foundation of China (31500499) and the Program for Science Technology Innovation Talents in Universities of Henan Province (16HASTIT019).
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 role of miR399 in mediating P transport and plant growth, transgenic *Arabidopsis* with *cL-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 *cL-miR399* were characterized. These results could deliver a new insight into the function of *cL-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 relative 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 KH₂PO₄ (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 *cL-miR399* and miR399a-f mature sequences were performed using the DNAMAN software.

To define target genes of *cL-miR399*, the mature sequence of *cL-miR399* was used as a query to search against the Chinese fir miRNA 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 *cL-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'-GGGGATAATTACTGGGGACTCTTC-3'; reverse, 5'-AGAAACAATTGCAGCGCAACTCTCT-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 *cL-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 *cL-MIR399* and PHO2, two kinds of *Agrobacterium* suspensions were mixed at a 1:1 ratio before infiltration of tobacco leaves.

**Cloning and overexpression of *cL-MIR399* in *Arabidopsis***: To overexpress *cL-MIR399* in *Arabidopsis*, pre-miR399 was cloned via reverse transcription PCR amplification using primers 5’-GGGGATAATTACTGGGGACTCTTC-3' and 5’-AGAAACAATTGCAGCGCAACTCTCT-3'. The amplified fragments were sequenced and then subcloned into the binary vector pCAMBIA2300 between the KpnI and BamHI 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, T1 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 *cL-MIR399, APH2*, *PHT1, PHT2*, and *PHT3* (Table 1 Suppl.). Relative expressions of different genes were normalized against an internal reference gene *Arabidopsis tubulin* using the 2⁻ΔΔCt 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 cm³ of concentrated HNO₃ and 2 cm³ of 30 % H₂O₂ at 140 °C for 30 min. The digested solutions were adjusted to a volume of 25 cm³ 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 predicted protein (unigene84522) were predicted from the *C. lanceolata* mRNA transcriptome database based on psRNATarget analysis.

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 Agrobacterium-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

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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 | GGGGAUAAUUACUGGGGACUCUCUUCUUUGGCUAGGAUUAAUUCAAUCUCUAAC UCAUGUAAAUUGUUUGGUCGCCUGGCAAAGGAGAGAGUUGGCCUCUGCAAAUUGUUCU |
| 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   | predicted 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 ≤ 0.05.
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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 quantitative 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 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.

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. 5. Phosphorus 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 ≤ 0.05.

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.
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 predicted 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.
C. lanceolata, which were confirmed to be critical in 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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