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Characterization and primary functional analysis of *Pinus densata* miR171

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Abstract

The miR171 is a conserved microRNA (miRNA) family and has been shown to participate in plant growth and development. However, the precise function of *miR171* in *Pinus densata* remains largely unclear. Mature *miR171* sequence comparison revealed a high similarity between *Arabidopsis thaliana* and *P. densata* and the *pre-miR171* could fold into a characteristic stem-loop hairpin structure. Genes encoding *GRAS* (*GAI-RGA-SCR*) family transcription factors and actin binding protein were identified as targets of *pde-miR171* using a modified RNA ligase mediated 5' rapid amplification of cDNA ends (RLM-RACE). Furthermore, the interaction between *pde-miR171* and *Arabidopsis SCL6* (*SCARECROW-LIKE6*) was further validated through transient co-expression of both genes in *Nicotiana benthamiana* leaves. Next, results of real-time quantitative PCR demonstrated that the expression of *pde-miR171* was significantly up-regulated in *miR171*-overexpressing plants than in wild-type plants, which was inversely correlated with the expression of *Arabidopsis SCL6* genes. In addition, overexpression of *pde-miR171* in *Arabidopsis* induced larger leaves and earlier flowering under long-day conditions compared with the wild type. The findings presented here suggest that *miR171* derived from a *P. densata* precursor together with its target gene *SCL6* may play important roles in the regulation of primary root growth, leaf shape, and flowering time in plants.

Additional key words: *Arabidopsis thaliana*, *Nicotiana benthamiana,* RLM-RACE, SCARECROW-LIKE6.

Introduction

MicroRNAs (miRNAs) are a group of small, non-coding RNAs that negatively regulate gene expression by guiding target mRNA cleavage or translational repression at transcriptional and post-transcriptional levels (Bartel 2004, Voinnet 2009, Shivaraj *et al.* 2014). These small non-coding RNA molecules are approximately 20 - 24 nucleotides in length and exhibit an important function in regulation of many aspects of plant growth and development, such as phase transition, flowering time, leaf morphogenesis, and responses to biotic or abiotic stresses (Sunkar *et al.* 2006, Curaba *et al.* 2013, Qiu *et al.* 2015).

MicroRNA171 (miR171) family is one of the most ancient and well conserved miRNA families, which has been isolated from a large number of plant species from mosses to flowering plants (Axtell *et al.* 2008, Cuperus *et al.* 2011, Hofferek *et al.* 2014). This family is known to regulate members of *GRAS* (*GAI-RGA-SCR*) gene family of transcription factors *SCARECROW-LIKE6* (*SCL6)/SCL6-IV*, *SCL22/SCL6-III* and *SCL27/SCL6-II,* also known as the *HAM* (*HAIRY MERISTEM*) or *LOM* (*LOST MERISTEMS*), that play an important role in plant root development, leaf shape, phase transition, and flowering time (Wang *et al.* 2010, Curaba *et al.* 2013, Li *et al.* 2014, Huang *et al.* 2017). In *Arabidopsis*, the *GRAS* gene family members *SCL6-II*, *SCL6-III*, and *SCL6-IV* are predicted to be *miR171* targets, and *SCL6-III* and *SCL6-IV* messenger (m)RNAs are confirmed to be targeted for mRNA cleavage by *miR171a* using 5'-rapid amplification of cDNA ends (5'-RACE) method. A recent study also found that *miR171* over-expressing

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Abbreviations: *GRAS* - *GAI-RGA-SCR*; mRNA - messenger RNA; miRNA - microRNA; RLM-RACE - RNA ligase mediated 5' rapid amplification of cDNA ends; *SCL6* - *SCARECROW-LIKE6*.
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transgenic *Arabidopsis* plants exhibit multiple developmental defects, where plant height, shoot branching, chlorophyll accumulation, primary root elongation, leaf shape, and patterning are altered (Wang *et al.* 2010). In barley, *miR171* acts mainly through the down-regulation of the *SCL6-II/III/IV* genes and over-expression of *hvu-miR171a* transgenic plants displayed a pleiotropic phenotype which included branching defects and late flowering, and also altered the vegetative to reproductive phase transition (Curaba *et al.* 2013). These results indicate that miR171 acts mainly by down-regulating *SCL6* genes to regulate a wide range of growth and developmental processes.

Pinus densata is an ecologically successful homoploid hybrid conifer with far-reaching evolutionary consequences (Wang *et al.* 2011). A genome-wide analysis of *miRNA* expression in *P. densata* was performed using high-throughput sequencing, and 19 conserved miRNAs from 14 families, including *miR160*, *miR164*, *miR165/166*, *miR167*, *miR171*, *miR172*, and *miR482* have been identified in our previous study (Wan *et al.* 2012). Furthermore, *miR171* and its target *SCL6* were found to be differentially expressed in needles, stems, and roots of *P. densata* by using real-time quantitative PCR (Qiu *et al.* 2016). Increasing evidence has demonstrated that investigating the expression patterns of *miR171* and its targets contributes to understanding the *miR171*-mediated regulation in *Arabidopsis*, barley, and tomato (Wang *et al.* 2010, Curaba *et al.* 2013, Huang *et al.* 2017). However, the underlying mechanism of regulation and relationship between *miR171* and *SCL6* is poorly understood in *P. densata.* Thus, the aim of present study was characterization of the relationship between *miR171* and *SCL6* and clarification of the biological role of *miR171* in conifers. The analysis of the *miR171* sequence and 5′-RACE (a modified RNA ligase mediated 5' rapid amplification of cDNA ends) showed that *miR171* could induce cleavage of the two predicted target mRNAs (*Unigene10015* and *Unigene83401*) in *P. densata*. To further investigate the role of *miR171* directly in the regulation of target genes and plant growth and development, transgenic *Arabidopsis* plants over-expressing *pde-miR171* were generated with *Agrobacterium*-mediated transformation. These experiments can serve as a foundation for further investigations of miRNA function in conifers.

Materials and methods

Plants and growth conditions: *Arabidopsis thaliana* L. ecotype Columbia (Col-0) was used as the wild type and for transformations. Sterilized seeds were sown on plates containing half-strength Murashige and Skoog (MS) solid medium. Plates were vernalized in darkness at 4 °C for 2 d and then transferred to a growth chamber under long-day conditions (a 16-h photoperiod with an irradiance of 120 μmol m^{-2} s^{-1}) and a temperature of 22 °C for 7 d. The seedlings (7-d-old) showing consistent growth were transplanted into soil and placed in a growth chamber under the same conditions described above until harvesting of siliques.

Freshly matured seeds were obtained from 30 homoploid hybrid *Pinus densata* Masters (*Pinaceae*) trees in Linzhi city (94.4 °E, 29.2 °N), Tibet, China. The collected seeds were used to grow small seedlings for total RNA extraction.

Nicotiana benthamiana Domin seedlings were grown in soil at conditions mentioned above.

Sequence retrieval and analysis: The hairpin structure of *pde*-miR171 precursor was predicted using the *RNAfold* web server (Zuker 2003; http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) with the default parameters. The mature *miR171* sequences of *Arabidopsis* were obtained from the *miRBase release 21* (http://www.mirbase.org, June, 2014). Mature *pde-miR171* and *Arabidopsis miR171a-c* sequences were aligned with *ClustalW2* program. To predict target genes of *miR171*, the mature sequence of *miR171* and 3 968 794 sequences in the *P. densata* mRNA transcriptome database were input into *psRNATarget* web server (http://bioinfo3.noble.org/psRNATarget/), and putative targets with expectation score ≤ 3.0 were selected (Zhang 2005). The sequences of *GRAS* gene family of transcription factors *SCL6-II* (At2G45160), *SCL6-III* (At3G60630), and *SCL6-IV* (At4G00150) were retrieved from the *Arabidopsis TAIR6* genome release (http://www.arabidopsis.org). Phylogenetic tree was constructed with a neighbor-joining method with 1 000 bootstrap replicates using *MEGA5.0* (Qiu *et al.* 2015).

Validation of miRNA target genes using RLM-RACE: A modified RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) was carried out to identify the cleavage sites of the predicted target mRNA using a 5′-RACE kit (*Takara*, Tokyo, Japan). Total RNA was extracted from 6-month-old *P. densata* seedlings using *Concert Plant RNA Reagent* (*Invitrogen*, Carlsbad, USA), following the supplied protocol. An RNA oligo adapter was ligated directly to the purified total RNAs (2 μg) without calf intestinal and alkaline phosphatase treatment. The cDNA samples were amplified by nested PCR according to the manufacturer's protocol. The initial PCR was carried out using the adaptor primer 1 (5′-primer) and a gene-specific outer primer. Nested PCR was carried out using 1 mm^3 of the initial PCR mixture, adaptor primer 2 (5′-nested primer), and a gene-specific inner primer

(Table 1 Suppl.). DNA bands with the expected sizes were gel-purified and cloned into a *pGEM T-easy* vector for sequencing. The sequenced DNA bands were identified as miRNA-guided cleavage products.

Transient co-expression of miRNA and its targets in *Nicotiana benthamiana*: *Pinus densata* genomic fragments forming fold-back structure for *pre-miR171* carrying the *miRNA* and/or *miRNA** duplex was amplified from 6-month-old *P. densata* with the primers listed in Table 1 Suppl. and cloned into a binary vector (pCAMBIA2300) under the control of 35S promoter. Its corresponding targets (*SCL6-II*, *SCL6-III*, and *SCL6-IV*) were amplified from *Arabidopsis* with the primers listed in Table 1 Suppl. and were inserted into the same vector. The cultures harboring *MIR171* from *P. densata* and *SCL6-II*, *SCL6-III*, and *SCL6-IV* from *Arabidopsis* were infiltrated separately or co-infiltrated into four-week-old *N. benthamiana* leaves as described by Zheng *et al.* (2012). For co-expression analysis, equal amount of *Agrobacterium* culture containing *pde-MIR171* and their targets were mixed before infiltration into *N. benthamiana* leaves.

Plasmid construction and the generation of transgenic *Arabidopsis* plants: A 187 bp genomic sequence containing *pre-miR171* fold-back was PCR amplified from genomic DNA isolated from six-month-old *P. densata* by using the specific primers (5'-GCA GATGGTACAGTGTTGTAAACGC-3' and 5'-AAT GGAATGGAATGGAGTGGATGAA-3'). The amplified fragments were sequenced and subcloned into *Kpn*I and *Bam*HI sites of pCAMBIA2300 downstream of the Cauliflower mosaic virus (CaMV) 35S promoter to generate the 35S:MIR171 construct. The construct was transferred into *Agrobacterium tumefaciens* strain EHA105 and then transformed into wild-type *Arabidopsis* plants using the vacuum infiltration method (Bechtold and Pelletier 1998). The transgenic lines were screened using kanamycin and confirmed by PCR amplification. Subsequently, gene expression analysis and phenotypic characterization were carried out using homozygous lines from T_3 unless otherwise indicated.

RNA isolation and gene expression analysis by quantitative real-time reverse transcription qPCR: Total RNA was extracted from 3-week-old transgenic *Arabidopsis* plants using *TRIzol* reagent (*Invitrogen*) according to the instructions. Reverse transcription reactions were performed using *Superscript II* reverse transcriptase according to the manufacturer's instructions using 2 μg of total RNA and oligo (dT) primer. Quantitative PCR was carried out on a *Rotor-Gene 3000* real-time PCR detection system (*Qiagen*, Germany) using *SYBR Green PCR Master Mix* (*Tiangen*, Beijing, China). The primers of *pde-miR171* were designed according to the sequence of *pre-miR171*, and the primers for the miR171 and its targets are shown in Table 1 Suppl. PCR amplification reactions were performed in a 20 mm^3 of mixture containing 2 mm^3 of diluted cDNA, 0.3 μM each primer, and 10 mm^3 of the *Thunderbird SYBR Green PCR Master Mix* with the following cycling conditions: 95 °C for 2 min, 40 cycles at 95 °C for 5 s, 60 °C for 15 s, and 72 °C for 10 s. After the PCR amplification, a thermal denaturing cycle at 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s was carried out to determine the melting curves and verify the specificity of the amplifications. The experiment was carried out with at least three independent replicates, and the *Arabidopsis tubulin* gene (accession numbers At5G62690) was used as an internal standard control. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) was used to calculate the relative expression of miR171 and its targets in the wild type was arbitrarily set as 1. The statistical analysis was performed using *SPSS 20* software (*SPSS*, Chicago, USA) with Duncan's multiple range test at the 5 % level of significance.

Results and discussion

The MiR171 family is one of the most conserved miRNA families in the plant kingdom and *pde-miR171* has been identified in *Pinus densata* by high-throughput small RNA sequencing in our previous study (Wan *et al.* 2012). The mature sequences of *Arabidopsis miR171a-c* were retrieved from *miRBase release 21* (http://www.mirbase.org) and subjected to multiple alignment with *P. densata* mature *miR171* using the *ClustalW2* program. Through the multiple sequence alignment, we found that the mature sequence of *pde-miR171*, which was 21-bp long (5'-UGAUUGAGCCGUGCCAAUAUC-3'), was highly similar to *Arabidopsis* mature *miR171a* sequence containing only one nucleotide different from it (Fig. 1*A*), indicating that the miR171 family sequence is indeed highly conserved. Furthermore, a 96 bp genomic sequence containing a *miR171* precursor was isolated from 6-month-old *P. densata* by PCR and it could fold into a characteristic plant *miRNA* stem-loop hairpin

Table 1. Predicted targets of *miR171* and their putative functions.

miRNA	Target genes	Score	Predicted function
pde-miR171	*Unigenes10015*	0.5	GRAS family transcription factor
	Unigenes83401	3.0	actin binding protein

structure as predicted by *MFOLD* (Fig. 1*B*). To predict the potential target of *miR171*, the *psRNATarget* web server (http://bioinfo3.noble.org/psRNATarget) was used. Two potential target genes, *GRAS* family transcription factor (*Unigene10015*) and actin binding protein (*Unigene83401*) that plays a role in cell division and plants development (McCurdy *et al.* 2001), were identified from *P. densata* mRNA transcriptome database (Table 1). In agreement with these results, Huang *et al.* (2017) demonstrated that *miR171* could target *GRAS* gene family of transcription factors S*CL6-II*, *SCL6-III*, and *SCL6-IV* in tomato. Similar results were also found in *Arabidopsis,* black pepper, and barley (Asha *et al*. 2013, Curaba *et al.* 2013, Ma *et al.* 2014). Cleavage of the target mRNA is the principal mode of regulation by plant miRNAs and a 5′ RACE assay is the most useful method to detect *in vivo* products of the miRNA mediated cleaved mRNA (Fan *et al.* 2015, Li *et al*. 2016). To further determine whether *miR171* could induce cleavage of the two predicted target mRNAs in *P. densata*, we amplified the cleavage products of *Unigene83401* and *Unigene10015* mRNAs in 6-month old *P. densata* seedlings using RLM-5'-RACE. As shown in Fig. 2, *Unigene10015* encoding *GRAS* family transcription factor had a cleavage site at the 13th nucleotide of *pde-miR171* from the 5'-end and *Unigene83401* encoding actin binding protein was sliced 20 nucleotides down-stream the canonical cleavage site, which could be attributed to secondary small interfering RNA in the 21-nucleotide register with the cleavage site for miRNAs (Ronemus *et al*. 2006, Wan *et al*. 2012). These results indicate that *Unigene10015* and *Unigene83401* are the targets of miR171, and *miR171* regulation of *Unigene10015* and *Unigene83401* occurs in *P. densata*. The characterization of *miRNAs* and their targets will lay the foundation to unravel the complex miRNA-mediated regulatory networks controlling plant growth and development and other physiological processes in *P. densata*.

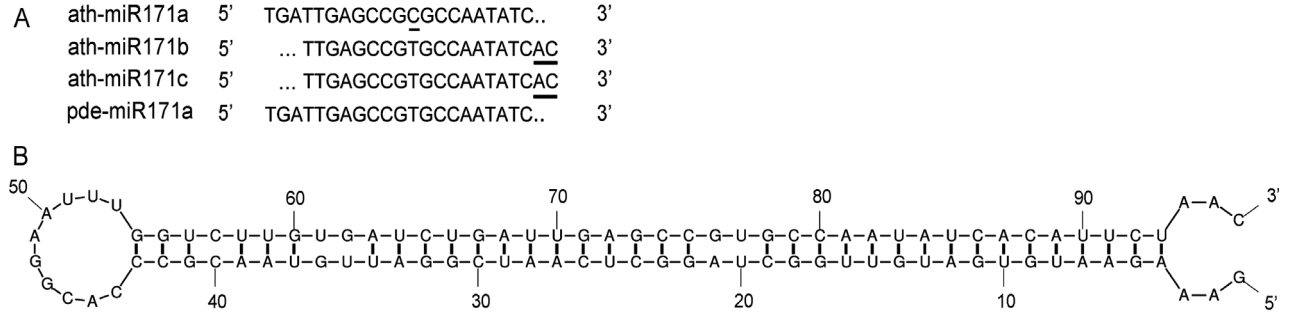

Fig. 1. Sequence analysis of *pde-miR171*. *A* - Sequence alignment of *Arabidopsis athmiR171a-c* (downloaded from *miRBase release 21*) and mature *pde-miR171*a. Mismatches are *underlined*. *B* - Hairpin secondary structure of the *pde-miR171* precursor.

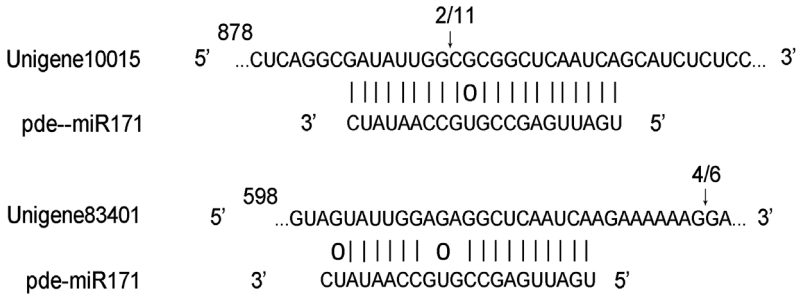

Fig. 2. Cleavage sites of the targets validated by 5' RACE. The predicted *mRNA* target (*top*) and its corresponding *miRNA* (*bottom*) are shown in each alignment, matches are indicated by *straight lines*, and G:U wobbles are represented by *ovals*. *Arrows* indicate the 5′ termini of *miRNA*-guided cleavage products identified by RLM-5'-RACE, and the numbers refer to the frequency of RLM-5'RACE clones corresponding to the cleavage sites.

Based on phylogenetic analysis, we found that *Unigene10015* was phylogenetically clustered into the *HAIRY MERISTEM* (*HAM*) subfamily of *GRAS* genes and showed the highest sequence identity with *Arabidopsis AtSCL6-III* (Fig. 3). To further evaluate if the three *SCL6* genes, *SCL6-II*, *SCL6-III*, and *SCL6-IV* from *Arabidopsis* are targets of *P. densata miR171*, we used an *Agrobacterium*-mediated transformation. We co-expressed *P. densata miR171* precursor and the three *Arabidopsis SCL6* target mRNAs in *N. benthamiana* leaf tissue. Transient co-expression of *miR171* and *AtSCL6-II* showed that the amount of *AtSCL6-II* was reduced by *miR171*. Similarly, *AtSCL6-III* and *AtSCL6-IV* expressions were very high in leaves infiltrated with

AtSCL6-III and *AtSCL6-IV* alone, but they were substantially reduced in the leaves where *miR171* and *AtSCL6-III* were co-expressed (Fig. 4), suggesting that *pde-miR171* can target and cleave *Arabidopsis* endogenous *SCL6*. Our results are supported by Wang *et al.* (2010), who demonstrated that *miR171* could cleave *SCL6-III* and *SCL6-IV* mRNA in *Arabidopsis* and tobacco leaves. Results present here provide evidence that *pde-miR171* has the capability to direct cleavage of *Arabidopsis* endogenous *SCL6 in vivo*.

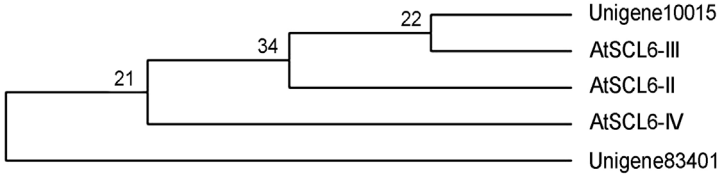

Fig. 3. Phylogenetic tree analysis of *pde-miR171* predicted targets (*Unigene10015* and *Unigene 83401*) and *Arabidopsis SCL6* genes. The sequences of *Unigene10015*, *Unigene83401*, *SCL6-II* (At2G45160), *SCL6-III* (At3G60630), and *SCL6-IV* (At4G00150) were aligned using *Clustal X*, and the tree was constructed by neighbor-joining method with 1 000 bootstrap replicates using *MEGA 5.0*.

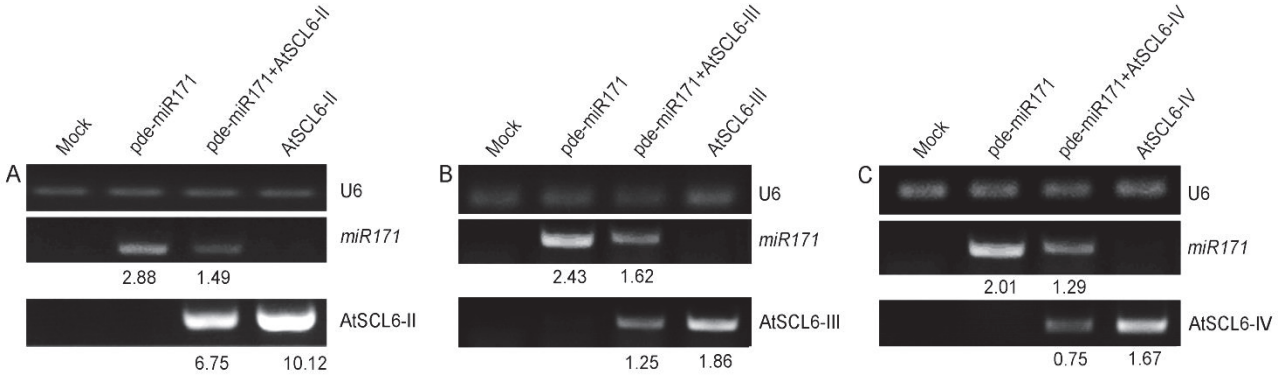

Fig. 4. Transient co-expression assay of *pde-miR171* and *Arabidopsis* targets in *Nicotiana benthamiana* leaves. Accumulation of mRNA of *pde-miR171* and/or *SCL6-II*, *SCL6-III*, and *SCL6-IV* is shown. Leaves transfected with an empty vector (Mock) were used as loading control. Band density ratios to U6 snRNA (U6) are shown under each panel.

In order to investigate the biological function of miR171 directly in plant development, we first generated transgenic *Arabidopsis* plants overexpressing the stem-loop precursor of *P. densata miR171* under the control of the enhanced CaMV 35S promoter. Among the generated independent transgenic lines, two lines were selected for analyses. The relative expressions of *pde-miR171* and *SCL6* were analyzed in *35S:MIR171* transgenic plants by real-time qPCR. The expressions of *pde-miR171* in transgenic line 1 and line 2 were higher than in the wild-type plants (Fig. 5). Moreover, the *pde-miR171* expression in the *35S:MIR171-2* line was higher than in the *35S:MIR171-1* line. Therefore, we selected *35S:MIR171-2* plants for further analyses. Next, we measured the abundance of *SCL6-II*, *SCL6-III*, and *SCL6-IV* by real-time qPCR, to examine whether these *SCL6* genes are down-regulated in *miR171*-over-expressing plants. We found that the expressions of these *SCL6* genes were significantly lower in the *miR171*-overexpressing plants than in the wild type (Fig. 5). This was consistent with interactions between targets and miRNAs, highly expressed miRNAs could cause the down-regulation of the corresponding target genes (Fan *et al.* 2015, Qiu *et al.* 2016, Huang *et al.* 2017). According to negative correlation between expression of *pde-miR171* and *GRAS* family transcription factor, it is reasonable to speculate that *miR171* derived from a *P. densata* precursor would negatively regulate *GRAS* family transcription factors *SCL6-II*, *SCL6-III*, and *SCL6-IV* to control a wide range of developmental processes in *P. densata*.

Most of plant miRNA functions have been deduced from overexpression of precursor sequences encoding miRNAs or gain-of-function mutants in which miRNA-resistant target genes are ectopically expressed (Guo *et al.* 2005). Transgenic plants constitutively overexpressing specific *miR171* genes have been well analyzed in *Arabidopsis* (Wang *et al.* 2010), barley (Curaba *et al.* 2013), rice (Fan *et al.* 2015), and tomato (Huang *et al.* 2017). *Arabidopsis* plants overexpressing *miR171c* and *scl6-II scl6-III scl6-IV* triple mutant plants caused many alterations, such as in plant height, flowering time, leaf architecture, shoot branching, and primary root elongation (Wang *et al.* 2010, Huang *et al.* 2017). In the present study, transgenic *Arabidopsis* plants overexpressing *pde-miR171* displayed larger leaves (the length and width of the leaves were increased on average by 25 and 40 %, respectively; Fig. 1 Suppl.) and shorter

primary roots as compared to wide type. Our results are supported by the results of Wang *et al.* (2010), who suggested that primary root elongation is strongly suppressed in transgenic *Arabidopsis* plants overexpressing *miR171c*. In addition to altered leaf shape and primary root elongation, overexpression of *pde-miR171* caused that plants grew taller and developed flowers 8 - 10 d earlier than wild type plants under long-day conditions (Fig. 1 Suppl.). In agreement with these results, Huang *et al.* (2017) also showed that transgenic tomato plants overexpressing *sly-miR171* are taller and have earlier phase transition times. On the basis of these observations, we speculate that *pde-miR171* may play an important role in the regulation of primary root growth, leaf shape, and flowering time in *P. densata*. However, further experiments are required for a more comprehensive understanding the regulatory roles of *pde*-miR171 during plant growth and development.

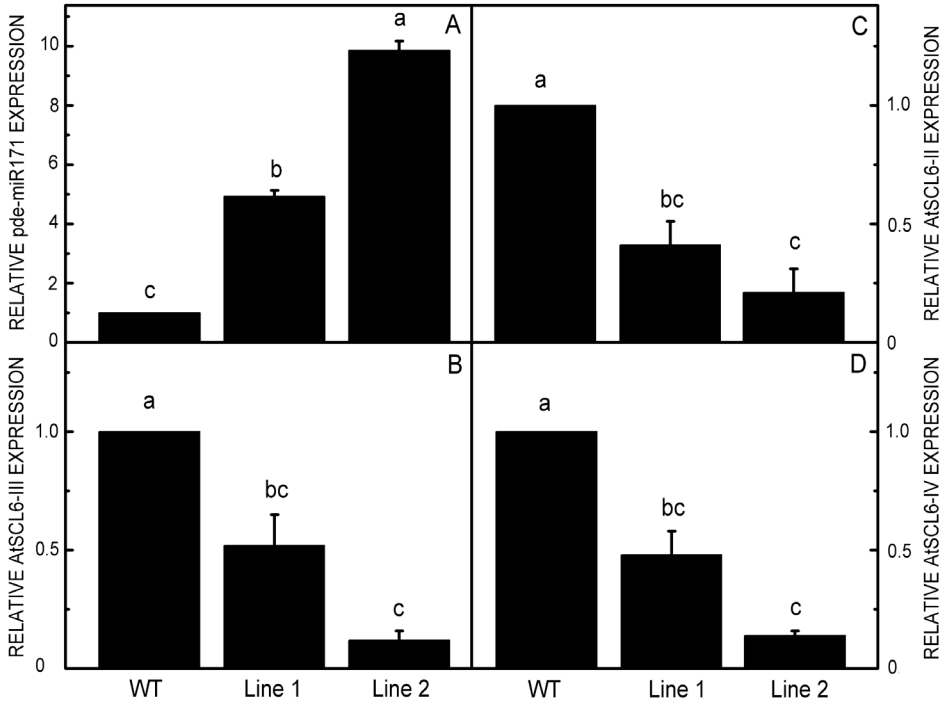

Fig. 5. Expression of *pde-MIR171* and *SCL6-II*, *SCL6-III*, and *SCL6-IV* in wild type (WT) and two transgenic 35S:MIR171 *Arabidopsis* lines (line 1 and 2). The expression of each gene in WT was arbitrarily set to 1. Means ± SEs, $n = 3$. Different letters indicate significant differences at the 0.05 significant level according to Duncan's multiple range test.

This is the first functional analysis of *P. densata miR171*. Taken together, this study demonstrated that *miR171* derived from a *P. densata* precursor together with its target genes *SCL6* may play a vital and potentially regulatory role in development of this important plant species. Comprehensive characterization of other *pde-miRNAs* and their targets would help understanding miRNA-mediated mechanisms controlling growth and development in conifers.

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