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Characterization and expression analysis of conserved miRNAs and their targets in *Pinus densata*

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that play crucial regulatory roles in diverse developmental processes *via* cleavage or translational inhibition of their target mRNAs. Although a growing number of miRNAs and their targets have been predicted and discovered *via* experimentation in many plants, little is known about conserved miRNAs and their target genes in *Pinus densata*. In the present study, the conserved miRNAs, miR171 and miR482, from *Pinus densata* were characterized. Analysis of miR171 and miR482 reveal that these miRNAs were highly conserved in other plant species. In addition, the precursors of miR171 and miR482 were validated by real time-PCR and sequencing. Using real-time quantitative PCR, miR171 and miR482 as well as their corresponding targets were found to be differentially expressed in needles, stems, and roots of *Pinus densata*. Furthermore two target genes, one GRAS family transcription factor protein gene and one nucleotide-binding site leucine-rich repeat (NBS-LRR) resistance protein gene, were experimentally verified to be the targets of pde-miR171 and pde-miR482, respectively, using RNA ligase-mediated 5'-rapid amplification of cDNA ends (RLM-RACE).

Additional key words: resistance protein, RLM-RACE, real time-qPCR, transcription factor.

Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs which are 20 - 24 nucleotides in length and possess regulatory functions at the transcriptional and/or posttranscriptional levels in most eukaryote organisms (Finnegan *et al*. 2003, Bartel 2004). They have received a great deal of attention over the past few years and have been found to play important roles in plant growth and development, including signal transduction (Jones-Rhoades *et al*. 2006, Chen 2009), maintenance of the floral and axillary meristems (Chen 2004, Chuck *et al*. 2009), as well as shoot and root development (Mallory *et al*. 2004, Subramanian *et al*. 2009). Recent studies have shown that miRNAs negatively regulate their target genes expression at the post-transcriptional and translational levels through mRNA cleavage or repression of translation, depending on the complementarity between miRNAs and their target genes (Voinnet 2009, Rogers *et al*. 2013).

Most pioneering studies on miRNA identification in plants have reported that a large number of known miRNAs are conserved in the plant kingdom (Reinhart *et al*. 2002, Asha *et al*. 2013). These conserved miRNAs share conserved functions in plant growth and development and stress responses among flowering plants. Highly conserved miRNA families, such as miR160, miR164, miR165/166, miR167, miR170/171, miR172, and miR482, suggest their evolutionarily conserved roles in plant development (Bonnet *et al*. 2004). Among the group of conserved miRNAs, two *Pinus densata* candidates (miR171 and miR482) were selected for this study. MiR171 belongs to the well conserved miRNA family known to regulate members of the GRAS gene family of transcription factors SCL6-II, SCL6-III, and SCL6-IV that play an important role in plant root and leaf development, lateral organ polarity, meristem formation, and vascular development (Wang *et al*. 2010). The conserved miR482 is ubiquitously distributed in gymnosperm, monocot, and dicot

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Abbreviations: miRNAs - microRNAs; MFE - minimal folding free energy; MFEI - minimal folding free energy index; NBS-LRR - nucleotide-binding site leucine-rich repeat; RLM-RACE - RNA ligase-mediated 5'-rapid amplification of cDNA ends; qPCR - quantitative PCR.

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plants and targets some nucleotide-binding site leucine-rich repeat (NBS-LRR) protein-encoding genes that play critical regulatory roles in disease resistance in plants (Zhu *et al*. 2013).

Pinus densata (Sikang pine) is an ecologically important conifer. It represents a highly successful case of homoploid hybrid speciation with far-reaching evolutionary consequences (Wang *et al*. 2011). Our work was enabled by the recent completion of a high-throughput sequencing miRNAs isolated from *P. densata* seedlings (Wan *et al*. 2012). Although Wan *et al*. (2012) identified 19 conserved miRNAs from 14 families in *P. densata* using high-throughput sequencing, these conserved miRNAs and their targets needed to be further validated and characterized by detecting and quantifying their expression in different tissues. Little is known about experimental identification of conserved miRNAs in *P. densata*, and much less is known about their target genes. Therefore, characterization and experimental validation of previously identified but un-validated miRNAs and their targets in *P. densata* could improve our understanding of the possible roles miRNAs have in regulating the growth, development, and other aspects in conifers. This study is mainly aimed at the characterization of *Pinus densata* miR171 and miR482. Subcloning and sequencing were conducted to further confirm the pre-miRNA sequences. Moreover, two target genes were experimentally verified by detection of miRNA mediated mRNA cleavage sites in *P. densata* mRNAs using RNA ligase-mediated 5'-rapid amplification of cDNA ends (RLM-RACE). Meanwhile, using real-time quantitative PCR (qPCR), the expression profiles of miR171, miR482, and the corresponding targets in *P. densata* were examined.

Materials and methods

Plants and the total RNA isolation: *Pinus densata* Masters seedlings were grown under standard glasshouse conditions (natural irradiance, a relative humidity of about 70 %, and day/night temperatures of 25/18 °C). Fresh needles, stems, and roots of two-month-old young seedlings were harvested and stored at -80 °C until use. The total RNA was extracted using the *Concert* plant RNA reagent (*Invitrogen*, Carlsbad, USA), following the supplied protocol. Agarose gel (1.0 %, m/v) electrophoresis was used to test RNA integrity as well as content, which was accurately determined with a *NanoDropTM 1000* spectrophotometer (*Thermo Fisher Scientific*, Wilmington, USA).

Subcloning and sequencing pre-miR482 and pre-miR171 sequences: A cDNA was synthesized from 2 μg of the purified total RNA in a 0.025 cm^3 reaction mixture, containing 200 U of *M-MLV* reverse transcriptase (*Promega*, Madison, USA) and 1 μg of random nonamer, according to the manufacturer's protocol. The housekeeping gene *Actin* was used as a positive control. The primers for pre-miRNA sequences are shown in Table 1 Suppl. Amplifications by PCR were carried out using the following thermal cycling conditions: 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 55 °C or 60 °C for 15 s, and 72 °C for 30 s. Amplification fragments were separated in a 2 % agarose gel with ethidium bromide staining. The gel-purified PCR fragments were subcloned into a *pGEM T-easy* vector (*Promega*) and sequenced.

Prediction of miRNA targets: To identify putative targets of miR171 and miR482, we used mature miR171 and miR482 as custom miRNAs as well as 3 968 794 sequences in the *P. densata* mRNA transcriptome database as custom mRNAs to search for complementary hits using the *psRNATarget* web server (http://bioinfo3.noble.org/psRNATarget/index.php?function=function3) with default parameter settings (Zhang 2005). Sequences with a penalizing score ≤ 3 were chosen as putative targets. We further performed *BLASTx* searches against the *NCBI* database to identify putative gene homologs. Similarities with an E-value of less than e^{-10} were considered a hit. Gene ontology analysis was also performed for gene annotation of predicted targets.

Validation of miRNA target genes using RLM-RACE: A modified RLM-RACE was performed for mapping internal cleavage of the predicted target transcripts using a 5'-RACE kit (*TaKaRa*, Tokyo, Japan) with slight modifications (Song *et al.* 2010). In brief, the purified total RNA (2 μg) was directly ligated to an RNA oligo adapter without calf intestinal and alkaline phosphatase treatment. Two pairs of nesting and nested gene-specific primers were designed and applied for PCR amplifications (Table 1 Suppl.). Bands of DNA with expected sizes were gel purified and cloned into a *pGEM T-easy* vector for sequencing. The sequenced DNA fragments were identified as miRNA-guided cleavage products.

Expression analysis of pre-miRNAs and their targets by real time qPCR: The total RNAs were isolated from needle, root, and stem tissues as described above. Reverse transcription was performed using *Superscript II* reverse transcriptase (*Invitrogen*) according to the manufacturer's instructions, using 1 μg of the total RNA and oligo (dT) primers. The *SYBR Green* real time qPCR was conducted on a *Rotor-Gene 3000* real-time PCR detection system (*Qiagen*, Germany). The primers of miR171 and miR482 were designed according to the sequence of pre-miRNA, and the primers for the miRNAs as well as target genes are shown in Table 1 Suppl. A quantitative PCR reaction

was conducted in 0.02 cm^3 of a mixture containing 0.002 cm^3 of a diluted cDNA, 0.3 μM of each primer, and 0.01 cm^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 15 s, 60 °C for 15 s, and 72 °C for 15 s. After 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 dissociation curves and verify the specificity of the amplifications. All the reactions were performed in three biological replicates, and the results were expressed relative to the expression levels of an internal reference gene, glyceraldehyde 3-phosphate dehydrogenase (CV170251), in each sample using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The expressions of miRNAs and their target genes in the root were arbitrarily set to 1.

Phylogenetic analysis: For phylogenetic analysis, we derived different precursor sequences of the miR171 and miR482 candidates from the miRBase release 21 (http://www.mirbase.org, June, 2014) and subjected them to a multiple alignment with *Pinus densata* pre-miRNAs using the *ClustalW2* program. The phylogenetic analysis was performed to understand the evolutionary relationships among them using the program *MEGA5.0*, and a phylogenetic tree was constructed using the neighbor-joining (NJ) method with 1 000 bootstrap replicates.

Statistical analysis: All data obtained were subjected to one-way analysis of variance (*ANOVA*) followed by Duncan's multiple range tests at a 5 % probability level using the *SPSS* software *v. 16.0*.

Results

The miRNAs are distinguished from other RNAs on the basis of the ability of their surrounding sequences to adopt a hairpin structure. Therefore, the corresponding precursor sequences of two miRNAs from the mRNA transcriptome database of *P. densata* were screened, and their secondary structures were predicted. The length of precursors to miR171 and miR482 were 96 and 98 nucleotides, respectively (Table 1). The sequences upstream and downstream of each mature miRNA were submitted to the RNAfold and all were determined to form stable stem-loop miRNA precursor structures (Fig. 1 Suppl.). The nucleotide precursor sequences miR171 and miR482 had an average A+U content of 53.65 and a minimum folding free energy (MFE) of -231.1 and -252.5 kJ mol^{-1}, respectively.

The precursor sequences of the pde-miR171 and pde-miR482 were further validated by PCR amplification and sequencing. The results of the verification of miR171 and miR482 (Fig. 1) show the amplification of the gene-specific DNA fragments of 96 and 98 bp. The amplification of the miR171 sequence was identical to the sequence obtained from *Illumina* sequencing (Wan *et al*. 2012), whereas the validated sequence of pde-miR482 had two mismatched nucleotides, which might be partially attributed to an assembly error during the *Illumina* sequencing process. Sequencing the amplified products of 96 and 98 bp further verified the exact precursor sequences of miR171 and miR482.

The identification of miRNA targets is an essential step towards the understanding of their regulatory functions. The target prediction of miR171 and miR482 was carried out using the Web-based program *psRNATarget* (http://bioinfo3.noble.org/psRNATarget/index.php?function=function3) using the default parameters. A total of 3 968 794 sequences from the *P. densata* mRNA transcriptome database and 2 mature miRNAs were used as a custom target database and as a custom miRNA database, respectively. Using a computational approach, a total of five potential mRNA targets were identified for two miRNA, most of which had

Table 1. Characteristics of precursor microRNAs (miRNAs) from *Pinus densata*. MFE - minimal folding free energy; MFEI - minimal folding free energy index.

miRNA	pde-miR171	pde-miR482
Mature sequence	UGAUUGAGCCGUGCCAAUAUC	UCUUUCCUACUCCUCCCAUUCC
Mature miRNA length [nt]	21	22
Precursor sequence	AAAGAAUGUGAUGUUGGCUAGGCUCAAUCG GAUUGUAACGCCCACGGAAUUUGGUCUUGU GAUCUGAUUGAGCCGUGCCAAUAUCACAUUC UAAC	AAGGCCAAUGGCUUGCGAGGGUAGGAAA AGCUCAGUGUGAUGAUAUAUUUCUCGCU CACUGAUCUGCAGUUUUUCCCACUCCUCC CAAGCCCAUGGCC
Pre-miRNA length [nt]	96	98
Arm location	3'	5'
MFE [kJ mol^{-1}]	-231.1	-252.5
A+U [%]	55.2	52.1
MFEI	1.28	1.04

Table 2. Predicted targets of miR171 and miR482 and their putative functions. *GO* - gene ontology.

miRNA	Target genes	Score	Predicted function	*GO* annotation
Pde-miR171	Unigenes10015	0.5	GRAS family transcription factor	DNA binding
	Unigenes84522	0.5	unknown	
	Unigenes83401	3.0	actin binding protein	actin binding
Pde-miR482	Unigenes7264	3.0	histone deacetylase	histone deacetylation
	Unigene45569	2.5	NBS-LRR resistance protein	defense response

homologs that have known functions in other species (Table 2). The pde-miR171 was predicted to target mRNA coding for a GRAS family transcription factor and an actin binding protein, whereas pde-miR482 targets presumably a histone deacetylase and an NBS-LRR resistance protein (Table 2).

The ability to detect the cleaved products of miRNA targets is necessary in the study of gene regulatory mechanisms of miRNAs. Experiments using RLM-RACE were performed to identify whether miR171 and miR482 could induce cleavage of the predicted target mRNAs. We identified the GRAS family transcription factor gene Unigene10015 as the target of miR171, and the NBS-LRR resistance protein gene Unigene45569 as the target of miR482 by detecting cleavage sites in the cDNA transcripts mediated by the miRNAs. As shown in Fig. 2, we found that miR482 cleaved the target with a high frequency (6 of 6 for miR482) between nucleotides 10 and 11 relative to the 5' end of complementary miR482. In addition, GRAS family transcription factor Unigene10015 in *P. densata* was a miR171 target and was cleaved three nucleotides downstream of the predicted site, which was likely due to further degradation by nucleases after miRNA-mediated cleavage (Wan *et al.* 2012). These results indicate that the targets identified using the internet are indeed genuine, this means that miR171 as well as miR482 can target and cleave the corresponding transcripts (Unigene10015 and Unigene 45569) in *P. densata*.

To investigate expression of pre-miRNAs in different tissues including roots, stems, and needles of *P. densata*, real time qPCR was used. The primers used were based on pre-miRNA sequences. Expression of

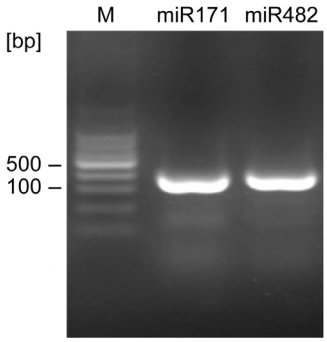

Fig. 1. Amplified pre-microRNA products of miR171 and miR482 using the subcloning method in a 2 % agarose gel with ethidium bromide staining. The total RNA was isolated from two-month-old seedlings of *Pinus densata.*

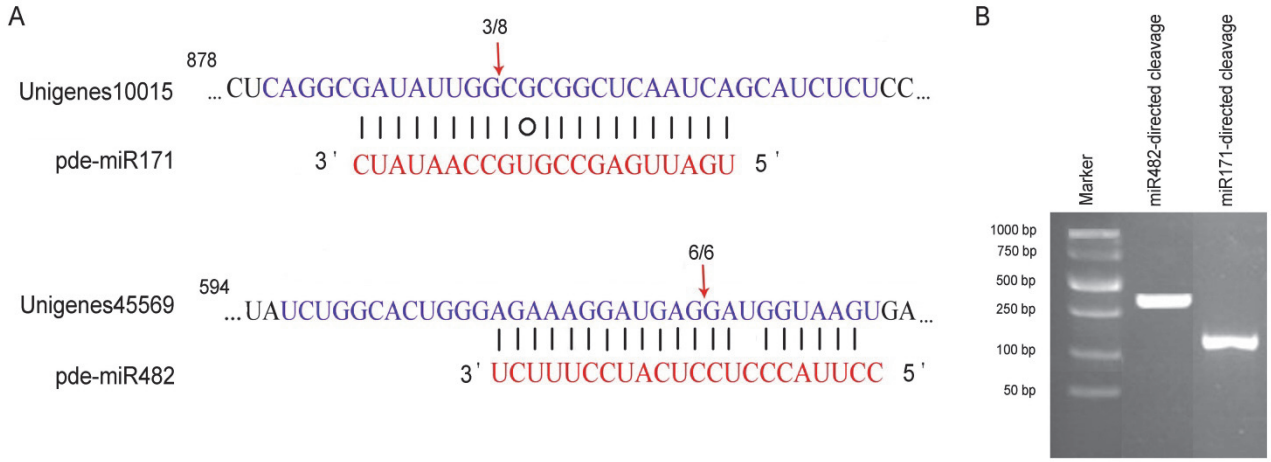

Fig. 2. Experimental validation of predicted mRNA targets for miR171 and miR482. *A* - The mRNA cleavage sites were determined by a modified 5'-rapid amplification of cDNA ends (RACE). A predicted mRNA target (*top*) and its corresponding microRNA (*bottom*) are shown in each alignment; matches are indicated by the *straight lines*, and a G:U wobble is represented by the *circle*. The *arrows* indicate the 5′ termini of microRNA-guided cleavage products identified by 5'-RACE with the frequency of clones shown. *B* - A gel image showing products of a 5'-RACE reaction which detected the miRNA-directed cleavage.

pre-miR171 was strongly detected in stems and needles, but pre-miR482 was expressed weakly in stems and needles (Fig. 3). The differential expression pattern provides information about the important functions of these miRNAs.

To further validate the role of miR171 and miR482 in regulating their target genes in *P. densata*, we investigated the transcription of the two identified target genes (Unigene10015 and Unigene 45569) using the real time qPCR method (Fig. 5). Expression of the GRAS family transcription factor (the target of miR171) was downregulated and showed a negative correlation with its corresponding miRNA in stems and needles (Fig. 4). Similarly, pre-miR482 expression was relatively low in stems and needles, and a high amount of its corresponding target transcript (Unigene 45569) was found. These results suggest miRNA-mediated regulation of their potential targets.

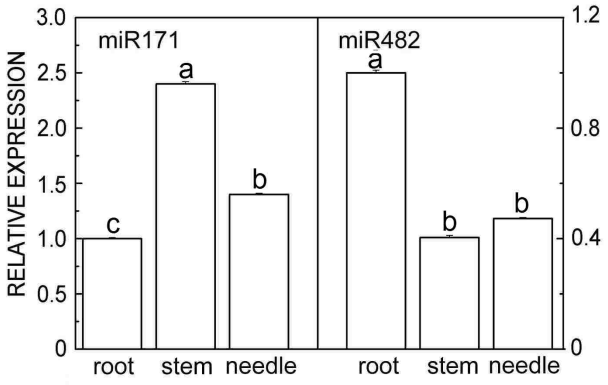

Fig. 3. Expression analysis of miR171 and miR482 in roots, stems, and needles of *Pinus densata*. The miRNA expression in roots was arbitrarily set to 1. Means ± SDs, $n = 3$ biological replicates. Different letters indicate significant differences at the 0.05 level.

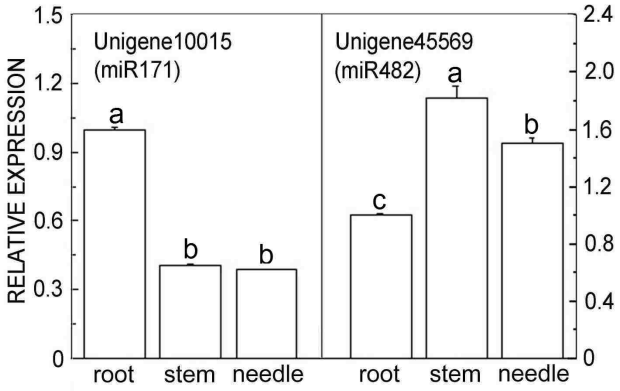

Fig. 4. Expression patterns of the target genes of miR171 and miR482 in roots, stems, and needles of *Pinus densata*. The miRNA target gene expression in roots was arbitrarily set to 1. Means ± SDs, $n = 3$ biological replicates. Unigene10015 and Unigene45569 are accession numbers based on the *Pinus densata* mRNA transcriptome database and represent the target genes of pde-miR171 and pde-miR482, respectively. Different letters indicate significant differences at the 0.05 level.

The mature sequences of miR171 and miR482 candidates from diverse plant species were downloaded from miRBase release 21 (http://www.mirbase.org, June, 2014) and subjected to multiple alignment with mature *P. densata* miRNAs using the *ClustalW2* program. *ClustalW2* alignment of *P.* densata miR171 and miR482 indicates that the sequences of miR171 and miR482 were highly conserved among plant species (Fig. 2 Suppl.). To further determine the phylogeny of the *MIR171* and *MIR482* genes, we selected different precursors of the miR171 and miR482 candidates from the species of dicot, monocot, gymnosperm, and lycophyte plants. From the phylogenetic relationships among the members of the two miRNA families, it is clear that *P. densata* is clustered with other gymnosperms [*Picea abies* (pab), *Pinus taeda* (pta)] in the phylogenetic tree for miR171 and miR482 (Fig. 5), in which angiosperms form a separate cluster indicating a deep evolutionary conservation of the miR171 and miR482 families. These results are consistent with early reports suggesting that a majority of plant miRNAs are conserved (Taylor *et al.* 2014). The identification of candidate miR171 and miR482 homologs from *P. densata* and their occurrence across different lineages of plants adds to evidence of the deep evolutionary conservation of the miR171 family and miR482 family in plants.

Discussion

The recently developed deep sequencing technology shows a great promise, it is able to generate an accurate and comprehensive picture of the small RNA transcriptome in different plants, tissues, and at different developmental stages (Song *et al*. 2010, Wei *et al*. 2011, Wan *et al*. 2012, Patanun *et al*. 2013, Qiu *et al*. 2015). Even though a computational approach has been demonstrated to be helpful, experimental validation must be performed to confirm the research *in silico*. In this study, we carried out subcloning experiments to validate the pre-miRNA sequences of miR171 and miR482, suggesting miR171 and miR482 indeed exist in the tissues of *P. densata*. In addition, the pre-miRNAs showed higher negative MFEs of -231.1 kJ mol^{-1} for miR171 and -252.5 kJ mol^{-1} for miR482. Furthermore, the pre-miR171 and pre-miR482 sequences could form hairpin structures as predicted by the RNA fold. Predicting the secondary structure of a pre-miRNA along with calculating the free energy are necessary for reducing the number of false positively identified miRNAs (Meyers *et al*. 2008, Yin *et al*. 2008). Due to the fact that the hairpin-loop secondary structure is not enough to distinguish miRNA from other types of coding or non-coding RNAs, the minimal folding free energy index (MFEI) is another useful parameter to distinguish miRNAs from other types of coding and non-coding RNAs (Patanun *et al*. 2013). A plant pre-miRNA should have a MFEI higher than 0.85, whereas other types of RNAs, mRNAs, tRNAs, and rRNAs, have lower MFEIs of 0.62 - 0.66, 0.64, and 0.59, respectively (Zhang *et al*. 2006). In the present study, pre-miRNA171 and pre-miRNA482 had high MFEI values (1.28 and 1.04), with an average of 1.16, suggesting that miRNA171 and miRNA482 could be present as real conserved miRNAs in *P. densata*.

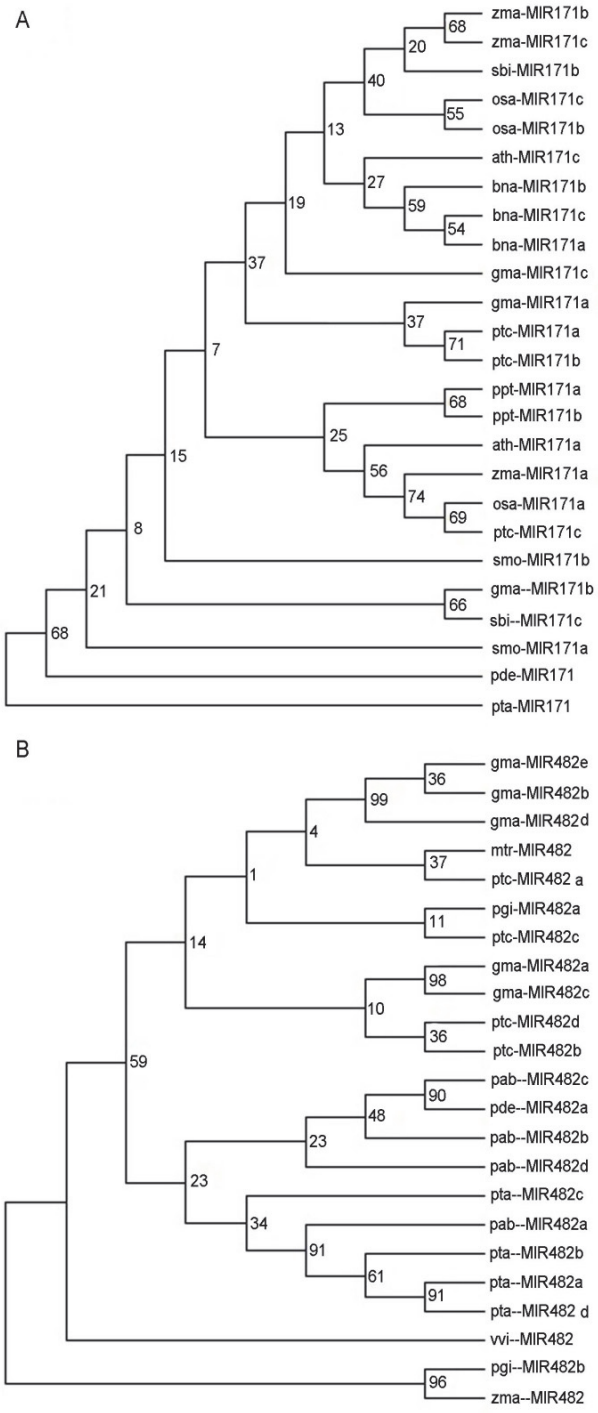

Fig. 5. A neighbor-joining phylogenetic tree of pre-miR171 (*A*) and pre-miR482 sequences (*B*) in dicot, monocot, gymnosperm, and lycophyte plants obtained from *MEGA 5.0*. Labels: pta (*Pinus taeda*), pab (*Picea abies*), pde (*Pinus densata*), cln (*Cunninghamia lanceolata*), smo (*Selaginella moellendorffii*), vvi (*Vitis vinifera*), sbi (*Sorghum bicolor*), gma (*Glycine max*), mtr (*Medicago truncatula*), ptc (*Populus trichocarpa*), bna (*Brassica napus*), ath (*Arabidopsis thaliana*), osa (*Oryza sativa*), zma (*Zea mays*), and pgi (*Panax ginseng*).

To provide clues about the physiological functions of small RNAs in *P. densata* development and growth, we utilized real time qPCR to validate the existence and different expression of pre-miRNAs and their target genes in roots, stems, and needles. Both miRNAs were amplified successfully from roots, stems and needles, but the expressions of pre-miR171 and pre-miR482 were different in the three tissues (Fig. 3). The pre-miR171 was expressed abundantly in stems and moderately in needles as compared to roots. The lowest expression of pre-miR171 being in roots is consistent with previous studies showing the highest expression of the target SCL6 mRNA in soybean (Li *et al*. 2010) and an inactive miR171 promoter in roots (Parizotto *et al*. 2004). In addition, Lu *et al*. (2005) reported that the expression of

miR482 is high in leaves and developing xylem tissues of poplar. However, our result shows that pre-miR482 had a week expression in stems and needles as compared with roots of *P. densata*. The difference in expression patterns of pre-miR482 suggests that the expression pattern may differ in diverse species despite the conserved nature of miRNAs (Yao *et al*. 2007).

To further understand the mechanisms of interaction between miRNAs and their target genes, the expression of the miRNA target genes (Unigene10015 and Unigene45569) were examined by real time qPCR in three tissues. As expected, the expression pattern of the target gene was negatively correlated with the expression of a given miRNA, which is in accordance with the gene silencing function of miRNAs (Wan *et al*. 2012). Quite typically, the expression of the GRAS family transcription factor (Unigene10015) was negatively correlated with the accumulation of pde-miR171, and the expression of pde-miR482 was also inversely correlated with its target gene, Unigene45569 (a NBS-LRR resistance protein), suggesting the miRNA-mediated regulation of their potential targets. Comprehensive characterization of miR171 and miR482 and their targets in different tissues would be helpful to understand the tissue-specific expression of the miRNAs as well as their regulatory roles with respect to different tissues and organs.

In order to investigate the function of miRNAs, the knowledge of miRNA target genes is essential for the understanding of the comprehensive central role of miRNAs (Sun 2012). Currently, the most efficient tool available for this purpose is the bioinformatics approach, which is based on a perfect or near perfect complementarity between miRNAs and their targets. Using the computational approach, we predicted five unigenes as putative targets for two conserved miRNAs in the present study. As expected, these target genes were similar or related to the previously validated plant miRNA targets in *Arabidopsis thaliana*, *Populus trichocarpa, Hordeum vulgare,* and *Pinus taeda* (Lu *et al*. 2005, 2007, Wang *et al*. 2010, Curaba *et al*. 2013).

A growing number of plant miRNA targets predicted through bioinformatics have been experimentally confirmed. Among the methods used to determine miRNA-dependent cleavage of targets, RLM-RACE is the most useful one to support bioinformatics data. We performed the 5'-RACE assays on the representative targets of the conserved miRNAs (the GRAS family transcription factor and NBS-LRR resistance protein targeted by miR171 and miR482). Disease-resistance proteins NBS-LRR could play an important role in the plant defense system or possibly act as receptors for sensing other extracellular cues (Lu *et al*. 2007, Shivaprasad *et al*. 2012). Lu *et al*. (2005) reported that miR482 was predicted to target putative disease resistance proteins in *P. trichocarpa*. Consistent with a previous finding, the NBS-LRR resistance protein was predicted to be the target of pde-miR482 in our present study. Furthermore, using 5'-RACE, the predicted target encoding a NBS-LRR resistance protein (Unigene 45569) was validated as a genuine target of pde-miR482, demonstrating that the disease-resistance gene targeted by tree-specific miRNAs may be an important component of the defence network for long-term adaptation of trees to pathogenic environments (Shivaprasad *et al*. 2012). In addition, the GRAS family transcription factor, which is involved in the regulating of plant growth and development (Wang *et al*. 2010), has been predicted to be the target of miR171 in *Arabidopsis* and barley (Wang *et al*. 2010, Curaba *et al*. 2013). Here by 5'-RACE approach, we found the GRAS family transcription factor to be the target of pde-miR171 that was negatively regulated by this miRNA through mRNA cleavage. One may speculate that miR171 acts by regulating the GRAS family transcription factor genes to control a wide range of developmental processes in *P. densata*.

Taken together, the results generated from this study shed more light on the existence and roles of *P. densata* miRNAs and their target genes in different tissues. Comprehensive characterization of conserved miRNAs and their target genes would facilitate a further understanding of the functions of small RNA-mediated regulation in conifers.

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