Transcriptome-wide Identification and Characterization of microRNAs and Their Targets in a Highly Adaptable Conifer *Platycladus orientalis*

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ABSTRACT. MicroRNAs (miRNAs) are short noncoding RNAs (20–25 nucleotides) that regulate gene expression posttranscriptionally. However, identification and characterization of miRNAs remain limited for conifer species. In this study, we applied transcriptome-wide miRNAs sequencing to a conifer species *Platycladus orientalis*, which is highly adaptable to a wide range of environmental adversities, including drought, barren soil, and mild salinity. A total of 17,181,542 raw reads were obtained from the Illumina sequencing platform; 31 conserved and 91 novel miRNAs were identified, and their unique characteristics were further analyzed. Ten randomly selected miRNAs were validated by quantificational real-time polymerase chain reaction. Through miRNA target predictions based on psRNATarget, 2331 unique mRNAs were predicted to be targets of *P. orientalis* miRNAs that involved in 187 metabolic pathways in KEGG database. These targets included not only important transcription factors (e.g., class III homeodomain leucine zipper targeted by por-miR166d) but also indispensable nontranscriptional factor proteins (i.e., por-miR482a-3p regulated nucleotide-binding site leucine-rich repeat protein). Interestingly, six miRNAs (por-miR16, -miR44, -miR60-5p, -miR69-3p, -miR166b-5p, and -miR395c) were found in adaptation-related pathways (e.g., drought), indicating their possible involvement in this species' stress-tolerance characteristics. The present study provided essential information for understanding the regulatory role of miRNAs in *P. orientalis* and sheds light on their possible use in tree improvement for stress tolerance.

miRNAs have been identified in more than 270 species that span the two major kingdoms of life: prokaryotes and eukaryotes (Kozomara et al., 2018). miRNAs are a group of endogenous small ribonucleic acid (sRNA) molecules, generally 20 to 25

nucleotides (nt) in length, which regulate target gene expression via the RNA interference pathway at translational repression or posttranscriptional degradation stage (Lagos-Quintana et al., 2001). The important miRNA roles have been discovered in regulating metabolism (Vienberg et al., 2017), tissue initiation and development (Xie et al., 2015), signal transduction (Fendler et al., 2011), cell cycle progression (Zheng et al., 2012), immune response (Chen et al., 2013), and response to different abiotic and biotic stress (Ferdous et al., 2015; Sun, 2012) of plants and animals. The pivotal roles of plant miRNAs have been demonstrated in different biological and metabolic processes including plant development, nutrient homeostasis, antibacterial reactions, phytohormones signaling, and response to stress (Ferdous et al., 2015; Liu and El-Kassaby, 2017; Qiu et al., 2016; Xie et al., 2015).

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In particular, a growing body of evidence suggests that miRNAs are involved in the responses to different stresses to conifers and other gymnosperms (Li et al., 2017; Liu and El-Kassaby, 2017). miRNAs generally bind to the 3'-UTR (untranslated region) of their corresponding target mRNAs and restrain protein production by destabilizing the mRNA and translational silencing and thus regulate the expression of specific genes. For example, the GRAS family protein and nucleotide-binding site leucine-rich repeat (NBS-LRR) resistance protein gene were key transcription factors for *Pinus densata* response to environmental stress, and their expression patterns has been experimentally demonstrated to be affected by miR171 and miR482, respectively (Qiu et al., 2016).

To date, there are no reports on miRNA from *Platycladus orientalis* (L.) Franco (Cupressaceae), an ecologically important conifer with high adaptability to a wide range of environmental adversities, including drought, barren soil, and mild salinity (Dong et al., 1990; Flora of China Editorial Committee, 1978; Hu et al., 2015, 2017). However, due to the limited genomic information, the physiological and genetic mechanisms of its adaptability are not yet well understood. Therefore, the identification of *P. orientalis* miRNAs is an important step to understand the regulatory networks in biological and metabolic processes. In general, the plant miRNAs often have been identified by two approaches: 1) experimental sequencing and 2) bioinformatic prediction. Using in silico prediction, miRNAs are identified by homology querying of various genomic resources (e.g., expressed sequence tags, genomic survey sequences, and high-throughput genomic sequences). Recently, the advent of high-throughput sequencing technologies has greatly accelerated the discovery of medium-to-low abundant and species-specific miRNAs from diverse plant species including conifer trees, such as *P. densata* (Wan et al., 2012), *Picea balfouriana* (Li et al., 2017), and *Picea glauca* (Liu and El-Kassaby, 2017).

The transcriptome dataset of *P. orientalis* generated by high-throughput sequencing technology has been published, and 148,867 unigenes were generated from terminal buds, microstrobilus, female strobilus, biennial leaves, and cambium tissues (Hu et al., 2016). This transcriptome dataset provides a useful reference sequence to identify new miRNA in *P. orientalis*, and these potentially discovered miRNAs will improve our understanding of the regulatory networks in this ecologically important species.

The main objective of this study was to identify miRNAs and their potential targets in *P. orientalis*, as well as a corresponding metabolic pathway involved in stress response. We constructed an independent *P. orientalis* sRNA library by high-throughput Solexa Illumina sequencing. The conserved miRNAs were then identified by the sequences blast in the public miRNA database, and novel miRNAs were identified according to previously published criteria for plant miRNAs (Meyers et al., 2008). Additionally, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to validate the accuracy and reproducibility of the newly identified *P. orientalis* miRNAs. This study formed a basis for further studying the regulative functions of miRNAs in the development and growth of *P. orientalis*.

Materials and Methods

PLANT MATERIALS. During the flowering season (April–May), terminal buds, microstrobilus, female strobilus, biennial leaves, and cambium tissues were sampled from five adult *P. orientalis* trees located in the Beijing Botanical Garden (Beijing, China).

All tissue samples were washing with tap water and deionized water to remove unwanted contamination, immediately frozen in liquid nitrogen, and stored at -80°C until further use.

SMALL RNA LIBRARY PREPARATION AND SEQUENCING. Total RNA was separately isolated from the tissue samples using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Equal quantities of total RNA from terminal buds, microstrobilus, female strobilus, biennial leaves, and cambium were pooled to generate an sRNA library using the TruSeq Small RNA Sample Prep Kit (Illumina, San Diego, CA). The polyacrylamide gel electrophoresis was used to enrich sRNA molecules in the range of 18 to 30 nt, which were ligated to adapters at 5' and 3' ends and amplified by PCR. After that, the products were purified and subjected to sequencing on Illumina HiSeq™ 2500 platform (Beijing, China).

SMALL RNA SEQUENCING AND DATA PROCESSING. After removing the adaptor sequences and poor-quality reads, high-quality sequences with 18 to 30 nt were reserved. All unique sequences were searched against the Rfam and National Center of Biotechnology Information (NCBI) noncoding RNA databases using the BLASTN program (Altschul et al., 1990), and ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA) as well as those containing poly-A tail were identified and removed from the sRNA library.

The sRNA sequences were then searched against known miRNA sequences in miRBase (Release 22.1), which contains 48,885 miRNA products from 271 species (Kozomara et al., 2018), to identify *P. orientalis* conserved miRNAs with less than two mismatches (Griffiths-Jones et al., 2008). The remaining reads that did not match any of the sequences in miRBase were used to predict novel miRNAs by Mireap (Li et al., 2012). Novel miRNAs were identified according to previously established criteria for plant miRNAs (Meyers et al., 2008). Because most of the pre-miRNA sequences are rapidly degraded after endonuclease Dicer-like cleavage, only the mature miRNA was obtained. Therefore, to produce a more comprehensive prediction for the miRNAs, publicly available RNA data of a closely related plant species (*Pinus taeda*) were also used as a reference to predict the secondary structure of *P. orientalis* miRNAs. If a reference sequence with miRNA-like alignment met the criteria (Meyers et al., 2008), it was considered a putative miRNA. Additionally, the secondary structure of the identified *P. orientalis* miRNAs will be validated by Mfold (Zuker, 2003).

CHARACTERIZATION OF CONSERVED *P. ORIENTALIS* MIRNAS. Among the group of conserved miRNAs, highly conserved miRNA families share conserved functions in plant growth, development, and stress responses. Therefore, the typically conserved *P. orientalis* miRNA families were selected for phylogenetic analysis to validate the conservation between the newly identified and already discovered members in a same miRNA family. We derived different precursor sequences of miRNAs from the miRBase release 22.1 (Kozomara et al., 2018) and subjected them to multiple alignment with newly identified *P. orientalis* pre-miRNAs using the ClustalW2 program (Larkin et al., 2007). The phylogenetic analysis was performed to determine the evolutionary relationships by the program MEGA5.0 (Tamura et al., 2011), and a phylogenetic tree was constructed using the neighbor-joining (NJ) method with 1000 bootstrap replicates.

QUANTITATIVE RT-PCR VALIDATION OF PARTIAL CANDIDATE MIRNAS. To confirm the accuracy and reproducibility of the identified miRNAs, 10 identified miRNAs were randomly selected,

and the same RNA preparation samples used in sequencing were subjected to qRT-PCR with U6 snRNA as reference (Bustin et al., 2009). Total RNA isolated from the collected plant materials was used for the first strand cDNA synthesis by FastQuant RT Kit (with gDNAase; Tiangen, Beijing, China) following the manufacturer's protocol. The primers for qRT-PCR were listed in Supplemental Table 1. qRT-PCR was performed using a SYBR Premix EX Taq Kit (TaKaRa Bio., Otsu, Japan) based on Applied Biosystems 7300 Sequence Detection System (Life Technology, Foster City, CA). The reaction system in a 20- μ L reaction volume containing 1 μ L diluted cDNA, 0.6- μ L primers, and 10 μ L 2 $\times$ PCR Master Mix (Tiangen). During the reaction, the fractional cycle number at which the fluorescence signal passes a fixed threshold is defined as threshold cycle (CT), and after the reactions were completed, the CT was automatically recorded. All reactions were performed with three replicates. The Δ Ct ($CT_{\text{sample}} - CT_{\text{ref}}$) was calculated for analyzing miRNAs expression level (Livak and Schmittgen, 2001).

PREDICTION OF miRNA TARGETS AND FUNCTIONAL ANNOTATION. The target genes of the new identified miRNAs were predicted using psRNATarget (Dai et al., 2018) with the following parameters: maximum expectation value at 3; target accessibility-allowed energy to unpair target site as 25; 17 bp upstream and 13 bp downstream for the flanking length around target site for target accessibility analysis; the range of central mismatch that will lead to translational inhibition is 10 to 11 nt; maximum mismatch at complementary site ≤ 4 without 0.5 gaps.

The putative functions of the identified miRNA target genes were assigned by performing BLASTX search against the non-redundant database of NCBI with an e-value of $1e-10$. To attain a better understanding of the miRNA function and unravel the miRNA gene regulatory network in terms of molecular function, cellular component, and biological process, gene ontology (GO) annotation and enrichment analysis were carried out by web-based BLAST2GO (Conesa et al., 2005). Finally, the metabolic pathways and their networks regulated by the potential miRNAs were identified through the target transcripts BLAST against Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Results

DEEP SEQUENCING. A total of 17,181,542 raw reads were obtained from the deep sequencing of the *P. orientalis* sRNA library. After removal of low-quality reads, adaptor sequences, and sequences with length outside of 18 to 30 nt, 7,616,082 clean reads, representing 1,091,289 unique reads, were reserved. The numbers and proportions of different types of sRNA are shown in Table 1. Among the unique clean reads, only 36.77% could be annotated, suggesting that the sRNAs have not been mined extensively in previous studies and that our study had greater potential to discover more miRNAs.

IDENTIFICATION OF *P. ORIENTALIS* CONSERVED MIRNAS. Thirty-one conserved miRNAs were identified comprising 18 miRNA families, the sequences of mature and precursor, and the predicted hairpin structures, shown in Table 2, Supplemental Table 2, and Supplemental Fig. 1, respectively. The length of *P. orientalis* mature miRNAs varied from 19 to 23 nt, of which 21 (41.2%) and 22 nt (35.3%) were the two major size classes (Table 2). The length of miRNA precursors ranged from 68 to 132 nt with an average value of 95. The minimal folding free energy (MFE) of *P. orientalis* miRNA precursors varied from -19.2 to -64.5 kcal/mol

Table 1. Distribution of *Platycladus orientalis* small ribonucleic acid (sRNA) among different categories.

Category	Unique sRNA		Total sRNA	
	Number	Proportion (%)	Number	Proportion (%)
Total	1,091,289		7,616,082	
rRNA ^z	383,368	35.13	4,829,219	63.41
tRNA ^y	168,03	1.54	276,215	3.63
snoRNA ^x	1,094	0.1	8,693	0.11
snRNA ^w	17	0	50	0
Annotated	401,282	36.77	5,114,177	67.15

^zRibosomal RNA.

^yTransfer RNA.

^xSmall nucleolar RNA.

^wSmall nuclear RNA.

with an average value of -43.9 kcal/mol. Additionally, each miRNA member had a different expression abundance from 2 to 8735, with an average expression value of 729 (Table 2).

In conserved miRNAs of *P. orientalis*, 15 new miRNA members belonging to 10 conserved families were the first reported in plant (Table 2). In addition, six (por-miR159a, -miR166a-5p, -miR166b-5p, -miR319a, -miR393a, and -miR1083a-5p) were further validated by qRT-PCR in the randomly selected miRNA validation experiment. Additionally, most of the identified conserved miRNAs of *P. orientalis* (81%) have been found in four other closely related conifer species; in particular, por-miR1314a, -miR159a, and -miR482a-3p were found in more than three conifers. Interestingly, one *P. orientalis* miRNA family may contain diverse members (e.g., the por-MIR166 family has six members), whereas only one member exists in 11 miRNA families (Supplemental Fig. 2).

PHYLOGENETIC ANALYSIS OF miR166 AND miR482. Among the conserved *P. orientalis* miRNAs, MIR166 is the most abundant miRNA family with six members and 10,723 expression abundances. This miRNA family is known to be drought-responsive and a regulator in axillary meristem initiation and leaf and vascular development in various plant species (Ferdous et al., 2015; Kantar et al., 2011; Sun, 2012). Additionally, miR482, another important stress-responsive miRNA, was identified; it is ubiquitously distributed in gymnosperm, monocot, and dicot plants and plays a critical regulatory role in disease resistance in plants (Zhu et al., 2013). A new member of miR482 was identified in our study, and its target was predicted in NBS-LRR protein-encoding genes, which is consistent with previous reports (Qiu et al., 2016). These two conserved and stress-responsive miRNA families were chosen for phylogenetic analysis not only to validate conservation between the newly identified miRNA members and previously discovered members in the same miRNA family, but also to determine the phylogeny of the stress-responsive miRNAs in this highly adaptive tree.

The miRNAs of angiosperm and gymnosperms always form a separate cluster, indicating the presence of deep evolutionary conservation inside of these two miRNA families. For example, miR166 is closely related to gymnosperms species (*P. taeda* and *P. densata*), and the precursor sequence of por-miR482a has shown similar evolutionary relationship with other gymnosperms species of *P. taeda*, and *P. densata* (Fig. 1). However, the homologs across different lineages of plants were also identified (e.g., por-miR482a-3p and zma-miR482; vvi-166d and por-miR166a-5p/3p), which further indicates that the ancient

Table 2. Conserved microRNAs (miRNAs) identified in *Platycladus orientalis*.

miRNA gene	miRNA sequence (5'-3')	Arm	miRNA length (nt) ^z	miRNA (no.)	Structures		Conserved in other species ^v				
					Reference ^y	MFE ^x (kcal/mol)	pta	pab	pde	smo	Total
por-miR156a	UUGACAGAAAGAUAGAGGGCAC	5'	22	369	Por	-43.2	+				34
por-miR156b	UGACAGAAAGAGAGUGAGCAC	5'	21	399	Por	-51.7	+				34
por-miR159a	UUGGAUUAGAGGGGCGUCU ^v	5'	19	1450	Por	-19.2	+		+		41
por-miR164a	UGGAGAGCAGGGCACGUGCG	5'	21	1541	Por	-42.4					33
por-miR166a-5p	GGAAUGUUGUCUGGCGUGCA ^v	5'	20	9	Por	-59.0					18
por-miR166a-3p	UCGGACCAAGGCUCAUUC	3'	20	142	Por	-59.0	+				29
por-miR166b-5p	GGAAUGUUGUCUGGCGUGCAU ^v	5'	21	2	Por	-45.7					16
por-miR166b-3p	UCGGACCAAGGCUCAUUC	3'	21	8735	Por	-45.7	+				29
por-miR166c	UCUCGGACCAAGGCUCAUUC	3'	21	1438	Por	-42.9	+		+		25
por-miR166d	CCGGACCAAGGCUCAUUC	3'	21	397	Pta	-42.0	+				33
por-miR171a	CGAGCCGAACCAUUAUACCC ^v	3'	22	20	Por	-38.6				+	21
por-miR319a	UUUGACUGAAGGAGCUC ^v	3'	21	102	Por	-64.5					34
por-miR393a	UCCAAAGGGAUUGCAUCGAUUC ^v	5'	22	32	Por	-37.3	+				31
por-miR395a	CAGAAAGUUUGGGGUACUC ^v	3'	21	73	Pta	-44.2			+		14
por-miR395b	CAGAAAGUUUGGGGAGAACUC ^v	3'	21	4	Pta	-49.2			+		36
por-miR395c	CAGAAAGUUUGGGGGACU ^v	3'	20	14	Pta	-50.2			+		3
por-miR396a	GUUCAAGAAAGCCGUGGAA ^v	3'	21	47	Por	-37.6			+	+	9
por-miR396b	GUUCAAGAAAGCUGUGGAAA	3'	22	92	Por	-47.1			+	+	39
por-miR396c	UUCACAGCUUUCUUGAACU	5'	21	36	Por	-42.5		+	+		48
por-miR398a	UGUGUUCACAGGUCACCCUG	3'	20	21	Por	-40.5	+				37
por-miR399a	UGCCAAAGGAGAGUUGCCUG	3'	21	32	Por	-43.5	+				29
por-miR399b-3p	UGCCAAAGGAGAGUUGCCUGCU ^v	3'	23	5	Por	-43.7					29
por-miR482a-3p	UCUUAACGAGUCCUCCCAUUC ^v	3'	22	404	Por	-50.1	+		+		32
por-miR1030a	UUUGCAUCUGCACCGCACCG ^v	5'	21	105	Por	-39.4					20
por-miR1083a-5p	UAGCCUGGAACGAGCACGC ^v	5'	20	1885	Por	-47.1	+			+	2
por-miR1314a	UCGGCCUUGAAUGUUAGGUGAG ^v	3'	22	32	Por	-54.4	+		+		4
por-miR2111a	AUAAUCUGCAUCCUGAGGUUG	5'	22	16	Pta	-45.6			+		19
por-miR2111b	UAAUCUGCAUCCUGAGGUUG	5'	21	27	Pta	-39.2			+		19
por-miR2111c	UAAUCUGCAUCCUGAGGUUGA	5'	22	12	Pta	-35.7			+		19
por-miR2950a	UUCCAUCUCUUGCACACUGAAC	5'	22	8	Pta	-43.8			+		6
por-miR6725a	UGGCAUCUGUCGAGGUCAUCUA	5'	22	78	Por	-40.4					2

^zLength of the miRNA (nt).

^yPor and Pta are representing the precures came from *Platycladus orientalis* and *Pinus taeda*, respectively.

^xMFE is minimal folding free energy (kcal/mol).

^vpta = *Pinus taeda*, pab = *Picea abies*, pde = *Pinus densata*, smo = *Selaginella moellendorffii*.

^vNew member of corresponding miRNA family.

+ Indicates that a species has a corresponding homologous miRNA with *P. orientalis*. Total is the total number of species that have a homologous miRNA that corresponds with *P. orientalis*.

Fig. 1. A neighbor-joining phylogenetic tree of pre-miR482 (A) and pre-miR166 sequences (B) in dicot, monocot, gymnosperm, and lycophyte plants.

miRNA regulatory system is well developed in the common ancestors of gymnosperms and angiosperms (Axtell, 2008).

IDENTIFICATION OF NOVEL miRNAs OF *P. ORIENTALIS*. On the basis of the criteria developed by Meyers et al. (2008; see

Methods), 91 sRNAs were identified as novel miRNAs, comprising 77 families (Supplemental Table 3). The precursors of these potential novel miRNAs were formed by a proper secondary hairpin structure with MFE ranging from -18.4 to -59.8 kcal/mol,

Table 3. Orthologs of putative novel *Platycladus orientalis* microRNAs (miRNAs) conserved in other species.

miRNA	sequence (5'-3')	Len. ^z	Highest homology		Conserved in other plants ^w				
			Name ^y	Mismatched bases (no.) ^x	pta	pab	pde	smo	total
por-miR02	GGAUGAAUCUGAGAACAACAU	22	gma-miR398b-5p	7					4
por-miR08	CGCUGACAUUGGAGGCCGACG	21	pab-miR1314	4		+	+		2
por-miR10	CGUAAUGUUGGUUCGGUUCAGC	22	ppe-miR171d-5p	5					28
por-miR37	UUAGGGUUAGGAAGAAGAAUAAU	24	ath-miR399b	3		+			29
por-miR61b-3p	UUUUUCCUGCACCACCAUUC	22	ppe-miR482f	3		+	+		20
por-miR61a-3p	UUUUCUGAACCACCAUUC	22	mes-miR482	4		+	+		11
por-miR62a	UUUUCUCUGUACCACCAUUC	22	vvi-miR3633a-3p	7		+	+		8
por-miR63b-5p	CAUGGGAGGGUUGGCAAAAU	21	ppe-miR482b-5p	6					6
por-miR63b-3p	UCUUGCCAGUCCUCCAAUGCC	21	pgi-miR482a	4		+			12
por-miR63c-5p	CUUCGGGGGAAAGGAAAAAU	21	osa-miR2118	5	+	+	+		8
por-miR63c-3p	UUUUCUGAUUCCUCCGAAGCC	21	osa-miR2118	5	+	+	+		8
por-miR64b-3p	UCUACCCGACCCUCCCAUGCC	21	csi-miR482b-3p	4		+			12
por-miR68-5p	CCUCUCCUCAAGGUUUUC	20	ppt-miR477c	4					18
por-miR399b-5p	CAGGGGAACGCUUUUAGCAGG	23	ath-miR399b	3		+			29
por-miR1083a-5p	GUGUUUCGUUUCAGGCUUAUG	22	smo-miR1083	7		+		+	2

^zLength of the miRNA (nucleotides).^ygma = *Glycine max*; pab = *Picea abies*; ppe = *Prunus persica*; ath = *Arabidopsis thaliana*; mes = *Manihot esculenta*; vvi = *Vitis vinifera*; pgi = *Panax ginseng*; osa = *Oryza sativa*; csi = *Citrus sinensis*; ppt = *Physcomitrella patens*; smo = *Selaginella moellendorffii*.^xmis. = the number of mismatch base.^wpta = *Pinus taeda*; pde = *Pinus densata*.+ Indicates that a species has a corresponding homologous miRNA with *P. orientalis*. Total is the total number of species that have a homologous miRNA that corresponds with *P. orientalis*.

with an average of -34.8 kcal/mol (Supplemental Fig. 3). The expression abundances of the novel miRNAs are from 3 to 3936, with an average of 131; however, the expression levels of most (83%) of these novel miRNAs are far below this average value. Interestingly, eight (11%) novel miRNA families have more than one member (e.g., por-MIR64 family has five members, which is the largest family in novel miRNAs). Most importantly, 15 miRNAs were predicted with an antisense miRNA (miRNA*), including three conserved miRNAs (por-miR399b-3p, -miR482a-3p, and -miR1083a-5p) (Supplemental Fig. 4).

Among the novel miRNAs of *P. orientalis*, 73 (80%) and 18 (20%) miRNA's precursor sequences came from *P. orientalis* and *P. taeda*, respectively (Supplemental Table 3). To determine whether the novel miRNAs were conserved in other plants, all of the 91 novel miRNAs were used as query sequences and BLASTN searched at miRBase database. Interestingly, 27 novel (29%) miRNAs were found have homologs to other plant species, and all of their precursor sequences came from *P. orientalis*. However, of these, only 15 novel (16%) miRNAs have homologs with more than two species (mismatch bases between 3 and 7) (Table 3). On the other hand, homology was not found for the remaining 66 novel miRNAs, and this may indicate that they are *P. orientalis*-specific.

VALIDATION OF *P. ORIENTALIS* MIRNA. All the randomly selected 10 miRNAs of *P. orientalis* could be readily detected by qRT-PCR (Fig. 2), suggesting that they are bona fide miRNAs of *P. orientalis*. However, their expression level varied significantly, the average ΔCt of the selected novel miRNAs was 0.69, which was much lower than conserved miRNAs (the average $\Delta Ct = 13.99$). All the selected conserved miRNAs appeared to be highly expressed, suggesting their conservative and primary function in essential biological processes of *P. orientalis*. Given the random nature of miRNA selection, only two novel miRNAs

of *P. orientalis* (por-miRNA55 and -miRNA67-5p) were selected, but all appeared to be expressed at lower levels compared with other conserved miRNAs. This is consistent with previously reported studies indicating that the relative expression levels of nonconserved miRNAs are usually at lower levels with tissue or developmental phase-specific patterns (Ruan et al., 2009; Song et al., 2010). Our validated results suggest that RNA sequencing was capable of successfully discovering candidate miRNAs from *P. orientalis* with high accuracy and efficiency.

IDENTIFICATION AND ANNOTATION OF miRNA TARGETS. A total of 148,867 sequences from *P. orientalis* mRNA transcriptome database and 122 identified miRNAs in this study were used as custom target and custom miRNA databases in the web-based program psRNATarget. Finally, 2331 unique mRNAs were predicted to be targets of the 122 miRNAs (Supplemental Table 4), suggesting that most of the miRNAs were predicted to have more than one target and that one target could be regulated by several miRNAs. These target annotations show that 56.6% can be annotated in the Nr database, and they were involved in a

Fig. 2. Quantitative real-time polymerase chain reaction validation of the identified miRNAs in *Platycladus orientalis*. The ΔCt value means the relative expression level of the verified miRNA; Groups 1, 2, and 3 represent three independent biological replicates.

variety of physiological processes in plants (Supplemental Table 4). Approximately 11 (36%) conserved miRNA targets were homologous to previously validated or predicted miRNA targets in *Arabidopsis thaliana* (Hsieh et al., 2009; Liu et al., 2008, 2009), *Oryza sativa* (Zhou et al., 2010), *Prunus persica* (Eldem et al., 2012), *Populus trichocarpa* (Shuai et al., 2013), *Medicago truncatula* (Wang et al., 2011), *P. densata* (Wan et al., 2012), *Triticum dicoccoides* (Kantar et al., 2011), *Gossypium raimondii* (Zhu et al., 2013), and *Glycine max* (Li et al., 2011; Xu et al., 2013) (Table 4). Additionally, most of these homology targets are plant-specific transcription factors and involved in stem and leaf development, flower and seed formation, root development, and the coordination of growth processes under adverse environmental conditions.

GO CLASSIFICATION AND KEGG PATHWAY ANALYSIS OF THE PREDICTED miRNA TARGETS. In the present study, $\approx 39.2\%$ targets could be annotated in GO database and categorized in terms of molecular functions (118 miRNAs), biological processes (117), and cellular components (112) (Supplemental Table 5). The GO enrichment analysis further disclosed that *P. orientalis* miRNAs was primarily involved with ATP binding (81), the nucleus (69), and the oxidation-reduction process (67) in categories of molecular functions, cellular components, and biological processes, respectively (Fig. 3). Interestingly, 27 and 22 miRNAs are significantly enriched in defense and stress responses, respectively.

Pathway-based analysis of the predicted miRNA targets shows that *P. orientalis* miRNAs are involved in 187 KEGG pathways (Supplemental Table 6), which could be assigned to five main categories of cellular processes (involved 19 miRNAs), environmental information processing (4), genetic information processing (27), metabolism (68), and organismal systems (28). We found that one miRNA may take part in several pathways and that one pathway could be regulated by several miRNAs. A more detailed distribution of miRNA in the subcategories is shown in Fig. 4, of which the carbohydrate metabolism is the subcategory that has been regulated most frequently by miRNAs, followed by global and overview maps (28), lipid metabolism (26), amino acid metabolism (25), signal transduction (23), and other functions.

GO annotation shows involvement of a number of miRNAs in response to different biotic and abiotic stresses. For example, GO enrichment found 20 miRNAs of *P. orientalis* (por-miR16, -miR22, -miR28, -miR32, -miR33, -miR35, -miR38, -miR39, -miR42, -miR57b, -miR59b, -miR60-3p, -miR60-5p, -miR61b-3p, -miR63c-5p, -miR67-5p, -miR396c, -miR396b, -miR1083a-5p, -miR6725a) involved in response to stress. Pathway-based analysis displayed a deeper insight into the associated metabolic and biosynthetic pathways with miRNA, such as por-miR396b play an important role in the pathway of plant hormone signal transduction (KEGG ID: ko04075) and -miR33 have a function in pathways of proteasome (ko03050), alpha-Linolenic acid

Table 4. Conserved *Platycladus orientalis* microRNA (miRNA) targets and their putative functions.

Target function ^z	miRNA	Target ^y	Species ^x	Source
MYB ^z transcription factors—ABA ^z response, NaCl stress response, floral	por-miR159a	comp61508_c0_seq2 (2.5)	Ath, Osa, Ppe	Eldem et al., 2012; Liu et al., 2008; Zhou et al., 2010
NAC ^z domain transcription factor—lateral root development	por-miR164a	comp70515_c0_seq2 (2) comp79385_c0_seq1 (2)	Mtr, Ptc	Shuai et al., 2013; Wang et al., 2011
HD-ZIPIII transcription factor—axillary meristem initiation, leaf and vascular development	por-miR166a-3p	comp53851_c0_seq2 (1) comp58082_c0_seq1 (1)	Ptc, Tdi, Gma	Kantar et al., 2011; Li et al., 2011;
	por-miR166b-3p	comp53851_c0_seq2 (1) comp58082_c0_seq1 (1)		Shuai et al., 2013
	por-miR166d	comp53851_c0_seq2 (0) comp58082_c0_seq1 (0)		
TIR1 and AFB2 and AFB3—susceptibility to virulent bacteria	por-miR393a	comp24630_c0_seq1 (1.5)	Ath, Ppe	Eldem et al., 2012; Liu et al., 2008
NBS-LRR protein-encoding genes—disease resistance in plants	por-miR482a-3p	comp86633_c0_seq1 (2.5)	Ath, Gra, Osa, Tdi, Pde, Ppe	Liu et al., 2008, 2009; Zhou et al., 2010; Kantar et al., 2011; Zhu et al., 2013; Eldem et al., 2012; Wan et al., 2012
Growth-regulating factor—stem, leaf development, growth processes	por-miR396a	comp46995_c0_seq1 (3) comp60601_c0_seq3 (3)	Ath	Liu et al., 2008, 2009
Kelch repeat-containing F-box protein—regulation of mineral homeostasis	por-miR2111a por-miR2111b por-miR2111c	comp72982_c0_seq1 (0.5) comp72982_c0_seq1 (0.5) comp72982_c0_seq1 (0.5)	Ath, Gma	Hsieh et al., 2009; Xu et al., 2013

^zMYB = myeloblasts; ABA = abscisic acid; NAC = *NAM* (“no apical meristem”), *ATAF1/2*, (“*Arabidopsis* transcription activation factor”), *CUC2* (“cup-shaped cotyledons 2”); HD-ZIP = class III homeodomain leucine zipper; TIR1 = transport inhibitor response1; AFB = auxin F-box protein; NBS-LRR domain protein = nucleotide-binding site leucine-rich repeat proteins.

^yAll predicted miRNA targets with penalty scores (shown in parentheses) of three or less are listed.

^xAth, Osa, Ppe, Mtr, Ptc, Tdi, Gma, Gra, and Pde are the abbreviations for *Arabidopsis thaliana*, *Oryza sativa*, *Prunus persica*, *Medicago truncatula*, *Populus trichocarpa*, *Triticum dicoccoides*, *Glycine max*, *Gossypium raimondii*, and *Pinus densata*, respectively.

Fig. 3. Gene ontology term enrichment analysis of the predicted targets. miRNAs = microRNAs.

metabolism (ko00592), and protein digestion and absorption (ko04974). Additionally, por-miR16, -miR44, -miR60-5p, -miR69-3p, -miR166b-5p, and por-miR395c also were identified in environmental adaptation pathway by KEGG annotation.

Discussion

The sRNA-mediated transcriptional or posttranscriptional regulation of gene expression, including the miRNA and siRNA

Fig. 4. Kyoto Encyclopedia of Genes and Genomes pathway analysis of the predicted microRNA (miRNA) targets of *Platycladus orientalis*. (A) Organism system, (B) metabolism, (C) genetic information processing, (D) environmental information processing, and (E) cellular processes.

pathways, which is an important epigenetic regulatory mechanism in plants. In this study, with the deep sequencing, a total of 1,314,450 unique reads were obtained from a stress-tolerant conifer, *P. orientalis*, and 31 conserved and 91 novel miRNAs were identified and characterized. Targets predicted and annotated shows that *P. orientalis* miRNAs regulate gene expression post-transcriptionally and act in a wide range of biological and metabolic processes, which is particularly useful for identifying specific miRNAs and pathways that related to adaptive mechanism of this stress-tolerant species.

The length of the identified mature and pre-miRNAs varied within a reasonable scope, which is in agreement with previous reports on gymnosperm, such as *P. glauca* (Liu and El-Kassaby, 2017), *P. balfouriana* (Li et al., 2017), and *P. densata* (Wan et al., 2012), and angiosperm plant that is, *A. thaliana* (Rajagopalan et al., 2006) and *P. trichocarpa* (Klevebring et al., 2009). The thermodynamical characteristic MFE is an important criterion for distinguishing miRNAs from other noncoding RNAs. The RNA secondary structure with lower MFE is generally more stable than those with higher MFE (Bonnet et al., 2004; Li and Zhang, 2011). The predicted *P. orientalis* pre-miRNAs have a low MFE with an average of -43.90 kcal/mol (Table 2, Supplemental Table 2). It is similar to the miRNAs in other plant species, such as *P. densata* and *M. truncatula* (Wan et al., 2012; Wang et al., 2011). Additionally, although most of conserved miRNAs of *P. orientalis* could be found in other plant species, the conservative is not always in conifers and other gymnosperms (i.e., por-miR164 is the first report in gymnosperms).

A phylogenetic study was useful to advance our insight into the evolutionary relationship of *P. orientalis* miRNAs with other plant species. The precursor sequences showed a high degree of similarity between the new identified member and other already-reported members in families of miR166 and miR482. In addition, it is clear that the precursor sequence of *P. orientalis* miRNAs could be clustered with other gymnosperms [i.e., *Picea abies* (pab), *P. taeda* (pta), *P. densata* (pde), *Selaginella moellendorffii* (smo)] in MIR166 and MIR482 phylogenetic trees, respectively (Fig. 1). This was consistent with previous reports that members within the same family are highly conserved among various species (Taylor et al., 2014).

The miRNA* is an incomplete complementarity single-stranded RNA of ≈ 22 nucleotide lengths and is produced in the process of synthesizing mature miRNAs. Previous studies have shown that the expression abundance of miRNAs* is much lower than the corresponding miRNAs (Berezikov et al., 2011; Blahna and Hata, 2013). In addition, the stability of the miRNA* 5' end is better than that of the 3' end of miRNA; this thermodynamic asymmetry makes miRNA more likely to be selected in the RNA-induced silencing complex, and the complementary miRNAs* are more likely to be degraded; therefore, miRNAs* have been considered ineffective in previous studies (Berezikov et al., 2011; Meng et al., 2011). However, with the in-depth research on miRNAs, several studies have found that miRNAs* are involved in the posttranscriptional regulatory network as miRNA. Recent studies have shown that the expression abundance of miRNA* is not lower than that of corresponding miRNAs in some biological tissues or is even much higher than miRNAs (Choo et al., 2014; Yonemori et al., 2016). In our study, there are also no certain expression patterns of the sense and antisense miRNAs in *P. orientalis*. For example, the expression abundances were 3936 and 19 for por-miR61a-3p/5p,

respectively, showing significant differences, but there seem to be no significant differences in por-miR68-3p/5p, with expression abundances of 14 and 18, respectively.

In this study, the transcription factor of the NAC domain and HD-ZIPIII were predicted to be targets of por-MIR164 and -MIR166, respectively, and these transcription factors were validated as playing important roles in root, leaf, vascular, and nodule development in *M. truncatula*, *P. trichocarpa*, and *T. dicoccoides* (Kantar et al., 2011; Shuai et al., 2013; Wang et al., 2011). Furthermore, NBS-LRR resistance-like protein, an important non-transcriptional factor protein, plays a critical regulatory role in disease resistance in plants (Eldem et al., 2012; Liu et al., 2008; Zhou et al., 2010); it also was predicted to be regulated by por-MIR482 in this study. These results show that *P. orientalis* miRNA targets encoded not only indispensable transcription factors but also nontranscriptional factor proteins involved in diverse physiological processes. Interestingly, the miRNA families MIR164, MIR166, MIR393, and MIR159 have been reported to be involved in physiological and biochemical metabolism of drought response in various species (Shuai et al., 2013; Wang et al., 2011), and these drought-related miRNAs were also found in *P. orientalis*, suggesting that *P. orientalis* may have its own defense systems to cope with adverse climatic conditions, and miRNA may play an important role in these metabolic systems.

A total of 187 KEGG pathways in *P. orientalis* were found to be regulated by miRNAs, providing a direction for studying the specific role of *P. orientalis* miRNAs, and especially for understanding the physiological and genetic mechanisms of the species' extraordinary adaptability to adverse environmental conditions. For example, six miRNAs targets were found to be involved in environmental adaptation pathways, including por-miR69-3p in circadian entrainment; por-miR395c in circadian rhythm; and por-miR16, -miR60-5p, -miR44, and -miR166b-5p in plant-pathogen interaction. Climate change has caused frequent drought stress at the flowering stage of *P. orientalis* in the northern region of China, which may result in declines of pollen fertility in *P. orientalis*. The flowering stage of *P. orientalis* is a period sensitive to environmental pressure, and therefore, the identified miRNAs related to adaptation are significant discoveries for regulating internal metabolism activity and improving adaptability. Additionally, pathway-based analysis could further our understanding of the biological functions and interactions of miRNAs, because the functional annotation results assigned to the same pathway may mean that these miRNAs were functionally related with each other. Therefore, our study opens a new avenue for studying specific miRNAs and pathways in this species, and have potential value in assisted breeding and conservation applications.

Conclusions

In this study, 31 conserved and 91 novel miRNAs in *P. orientalis* were identified and analyzed. Of these, 15 conservative miRNAs were discovered first; 15 miRNAs were found with an antisense miRNA; and the conservatism of newly identified and previously discovered members were detected by phylogenetic analysis in MIR166 and MIR482, respectively. Additionally, the accuracy and reproducibility of the identified *P. orientalis* miRNAs were validated by qRT-PCR. Target prediction and annotation showed that *P. orientalis* miRNAs involved in a wide range of biological and metabolic processes, which is particularly useful for understanding specific miRNAs and pathways

that are related to adaptive mechanisms of this highly adaptable species.

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