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Identification and Characterization of Novel MicroRNAs from *Populus cathayana* Rehd

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Abstract MicroRNAs (miRNAs) are highly conserved, endogenous, non-coding small RNAs of about 18–24 nucleotides in length. As negative regulators, miRNAs inhibit gene expressions at the post-transcriptional level by guiding cleavage or attenuating the translation of target genes, which affects organismal morphogenesis, development, and adaptability to changes occurring in the environment. Although miRNAs have been found in *Populus* species and have been shown to have diverse biological functions, few have been described in *Populus cathayana* Rehd. In this study, we have identified novel miRNAs from *P. cathayana* by constructing a library of small (18–24 nucleotides) RNA. After sequencing 691 small RNAs from *P. cathayana*, alignment to the *Populus* sequence database and secondary structure prediction were performed. These bioinformatic analyses identified five candidate miRNAs that were designated ptc-miR801, ptc-miR74, ptc-miR102, ptc-miR367, and ptc-miR213. Expression of these miRNAs was validated using real-time quantitative PCR analysis, and four of them displayed distinct expression patterns in leaves, stems, and roots. Our results show that four of the miRNAs are likely specific to leaves. Putative targets for these miRNAs play important roles in development and response to abiotic stress.

Keywords Novel miRNA · miRNA Library · *P. cathayana* Rehd · Expression · Target genes

Introduction

MiRNAs are highly conserved and endogenous 18–24 nucleotide (nt) molecules which exist extensively in plant organs (Ambros 1989; Bartel 2004; Chen 2005; Zhang et al. 2006a, b, 2007; Tong et al. 2007; Tang et al. 2003, 2008). In plants, miRNAs act as negative regulators, mainly by inhibiting gene expressions at the post-transcriptional level. In this mechanism, a miRNA combines with the ARGO NAUTE protein to form the miRNA silencing complex (miRISC) (Schauers et al. 2002; Bartel 2004; Griffiths-Jones 2004; Griffiths-Jones et al. 2005). With their complementary base pairing property, miRNAs induce the miRISC to target the corresponding mRNA, which then mediates irreversible cleavage or translational repression of target transcripts (Liu et al. 2005; Blow et al. 2006; Chu and Rana 2006).

Cloning and bioinformatic analysis have identified many miRNAs in a wide range of organisms from plants to humans (Bonnet et al. 2004; Jones-Rhoades and Bartel 2004). Large-scale sequencing approaches have been applied to discover small RNAs at the genome level (Lu et al. 2005a, b), and hundreds of miRNAs have been identified in different eukaryotic species. Recently, 10,581 miRNAs were published in the Sanger miRNA Database (miRBase release 14.0). It was estimated that the total number of miRNA genes represents approximately 1% of the expressed genome in complex organisms, implying that a myriad of miRNAs remain to be discovered and their roles in cellular processes such as development, morphogenesis, and adaptability to changing environments

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elucidated (Lai 2003; Lim et al. 2003; Bartel 2004). The first plant miRNAs were found in *Arabidopsis thaliana* by means of a library of small RNAs to identified 16 novel miRNA (Reihart et al. 2002). The majority of miRNAs in plants have been identified by cloning techniques or by computational prediction in *Arabidopsis*, *Oryza sativa*, and *Populus*, and miRNA families found in plants are more conserved when compared with those in animals (Axtell and Bartel 2005).

Recent studies have shown that miRNAs play critical regulatory roles in plant organ development, specifically patterning of the roots, stems, and leaves (Lai et al. 2003; Lewis et al. 2005; Zhang et al. 2006a, b). Under stress conditions, plants express specific miRNAs as a response (Sunkar and Zhu 2004; Sunkar et al. 2007; Lu et al. 2005a, b). It has been demonstrated that several miRNAs in plants regulate programmed development in conjunction with transcription factors. Furthermore, it has also been shown that miRNAs are involved in regulating additional physiological processes including low temperature (Sunkar and Zhu 2004; Lu et al. 2008; Zhou et al. 2008), salt (Sunkar and Zhu 2004; Borsani et al. 2005; Lu et al. 2008; Ding et al. 2009), heat (Lu et al. 2008), dehydration (Sunkar and Zhu 2004; Reyes and Chua 2007), oxidative stress (Sunkar et al. 2006), and mechanical stress (Lu et al. 2008). Sunkar and Zhu (2004) constructed miRNA libraries using *A. thaliana* from plants subjected to stress caused by drought, salinity, low temperatures, and abscisic acid. Northern blot analysis has shown that drought, low temperatures, and salinity affects expression of miR319c, miR393, miR395, miR397b, and miR402. MiR398 and its targets are differentially expressed in response to high salinity and other types of biotic and abiotic stressors (Jia et al. 2009). Some miRNAs in plants are specifically expressed in certain tissues. In a stress environment, plants express specific miRNAs as a response to the stimulation (Sunkar and Zhu 2004; Lu et al. 2005a, b). For example, miR397 is highly and specifically expressed in undifferentiated embryogenic calli from rice, while being expressed at low levels in differentiated calli and mature organs (Luo et al. 2006). Another example is *A. thaliana*, where the miR397 target *AtLAC15* is predominantly expressed in roots (McCaig et al. 2005) and is involved in root elongation and lignification (Liang et al. 2006).

Computational predictions of putative targets for miRNAs in plants have been carried out to a greater extent than in animals, due to their almost perfect complementarity to target genes (Rhoades et al. 2002). Specific genes have been predicted to be targets of stress-responsive miRNA families. The functions of these predicted target genes are diverse. For example, the pentatricopeptide repeat protein family, which is controlled by miR477 genes (Lu et al. 2005a,b), is involved in suppressing salt stress in rice (Baldwin and Dombrowski 2006; Ma et al. 2006). High concentrations of NaCl increases levels of laccase transcription in tomato root (Wei et al. 2000). The finger pattern, which is established by a combination of zinc finger proteins and Zn^{2+}, plays an important role in gene expression and regulation, cell differentiation, embryonic development, and also improves protein resistance to salinity stress. The miR398 and miR477 families regulate expression of the zinc finger proteins (Sunkar and Zhu 2004; Lu et al. 2005a, b).

Populus is one of the most important deciduous tree genera in worldwide plantations, and is characterized by rapid growth and regeneration. *Populus* has been used as a model plant for the forestry engineering research, due to its relatively small genome (480 Mb) (Tuskan et al. 2006). Moreover, the draft sequence of the *Populus* genome was completed in 2004 (Brunner et al. 2004). Among the five sections of *Populus* in China, *P. cathayana* Rehd is the largest and includes 34 species, 21 variations, and 4 forms. We have constructed a library of small RNAs from *P. cathayana* to search for novel miRNAs.

After sequencing and analysis of the library, 210 small probable RNAs were obtained. After homologous alignment to the *Populus* DB and secondary structure prediction, we obtained five candidate miRNAs and verified their expression profiles by real-time quantitative polymerase chain reaction (qRT-PCR) in *Populus*. Meanwhile, analysis of four of these miRNAs revealed distinct expression patterns in leaves, stems, and roots by qRT-PCR. The expression of miRNAs may be induced by salinity stress. Expression of two novel miRNAs was confirmed using qRT-PCR.

Materials and Methods

Plant Materials

Six-month-old *P. cathayana* plantlets were grown in Murashige–Skoog (Murashige and Skoog 1962) nutrient medium containing 3% (*w*/*v*) sucrose and 0.8% (*w*/*v*) agar at 25°C under a 16 h light/8 h dark cycle per day. For stress treatments, *P. cathayana* was subjected to a salt stress experiment. This experiment involved the application of salt by immersing plant roots in a water culture containing 100 mmol L^{-1} NaCl for 72 h.

RNA Extraction and Polyadenylation

P. cathayana plantlets were transferred into liquid nitrogen and ground to a fine powder, small RNAs (20–40 bp) were extracted using the mirVana™ miRNA Isolation kit (Ambion, USA). The concentration and purity of RNA were quantified by absorbance at 260, 280, and 320 nm.

The small RNAs were polyadenylated by poly(A) polymerase (Ambion, USA). According to the polyadenylation reaction, 50 μL was incubated with 10 μg of small RNAs and 5 U of poly(A) polymerase. The reaction was placed at 37°C for 1 h. After incubation, poly(A)-tailed RNA was purified using phenol: chloroform: isoamyl alcohol extraction (25:24:1) and phenol/chloroform (24:1) extraction. Next a 5′ RNA adaptor (5′-CGACUGGAGCACGAGGA CACUGACAUGGCU GAAGGA GUAGAA-3′) was ligated to the poly(A)-tailed RNA. 10 μL was incubated with 5 μL of poly(A)-tailed miRNA and 1 U of T4 RNA ligase (TaKaRa, Japan). The reaction was incubated at 37°C for 1 h. After incubation, poly(A)-tailed RNA was purified using phenol/chloroform/isoamyl alcohol extraction (25:24:1) and phenol/chloroform (24:1) extraction. The RNA was recovered by ethanol precipitation and was eluted into 20 μL DEPC water.

Reverse Transcription

A reverse transcription (RT) reaction was performed using 2 μL of poly(A)-tailed small RNAs, 1 μL of RT primer {5′-GCGAGCACAGAATTAAT ACGACTCACTATAGG-d(T)18V(A,G or C)N(A,G,C or T)-3′} with 1 U of SuperScript II (Invitrogen, USA). A 2 μL of tailed small RNAs were incubated with 1 μL of RT primer at 65°C for 5 min to remove any RNA secondary structure. The reactions were chilled on ice for at least 2 min and the remaining reagents (10× buffer, dNTP mix (10 mM each), RNaseout, $MgCl_2$, SuperScript II) were added as specified in the SuperScript II protocol. The reaction proceeded for 60 min at 42°C then the reverse transcriptase was inactivated by 15-min incubation at 70°C.

Primer Design, PCR, and Library Preparation

The primer sequences are only complementary to part of the miRNA gene 5′ end adaptor and 3′ end poly(A) tail. Amplification was carried out for 25 cycles with a final annealing temperature of 55°C, using 5′ primer (5′-GGACACTGA CATGGACTGAAGGAGTA-3′) and 3′ primer (5′-GCGAGCACAGAATTAATA CGAC-3′). The PCR products were analyzed on a 4% agarose gel. Gel slices containing DNA with a size of ~100 bp were excised and the DNA recovered by the column DNA back kit (TIANDZ, China). DNA fragments were directly subcloned into pMD19-T vector (TaKaRa, Japan) and sequenced on an ABI3730×l DNA sequencer. Since sequencing is not directional, we used the 3′ adapter sequence to determine the direction of each insert. Where necessary, sequences were converted to their corresponding reverse complements to facilitate computational analysis.

BLAST Analysis and Prediction of Stems-loop Structures

For computational analysis and abundance calculations, only reads with a recognizable adapter sequence were retained for further analysis. The *P. cathayana* small RNA library was used in BLAST searches to identify candidate *Populus* miRNAs. We screened the databases of *Populus* DB (http://www.populus.db.umu.se/blast.php), and the *Populus* genome database (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) for supplementary annotation of cloned sequences. Sequences of rRNAs, tRNAs, snRNAs and snoRNAs were downloaded from databases including the European ribosomal RNA database (http://www.psb.ugent.be/rRNA/, for rRNA), the Genomic tRNA database (http://lowelab.ucsc.edu/GtRNAdb/, for tRNA) and NONCODE (http://www.bioinfo.org.cn/NONCODE/, for snRNAs and snoRNAs). The remaining small RNAs were also analyzed for predicted secondary structure. Genomic regions containing sequences homologous to *Populus* miRNA candidate sequences, with approximately 300 bp of flanking sequence, were extracted and analyzed for potential to adopt a stem-loop conformation using RNA-fold software (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi) and RNA structure prediction software (http://microrna.sanger.ac.uk/sequences/). Candidate sequences were then screened against a database of known miRNAs (http://microrna.sanger.ac.uk/sequences/) to compare our candidate *Populus* miRNAs.

Primer and Analysis of miRNA Expression of qRT-PCR

Because of their small size, detection of miRNAs by traditional qRT-PCR is technically demanding. A poly(A)-tailed was added to the miRNA 3′ ends to increase their size. The qRT-PCR primer design is very important to ensure specificity of the PCR. For the 3′ primer, the sequence 5′-GCGAGCACAGA ATTAATACGAC-3′ used for miRNA amplification was unchanged. For most miRNAs, the 5′ primer sequence is the same as the miRNA gene (Table 1).

MiRNA expression was studied by qRT-PCR using the 2×QuantiTect SYBR green PCR kit (QIAGEN, Netherlands). Total RNA was extracted from *P. cathayana* and polyadenylated with poly(A) polymerase. RNA was reverse transcribed as described above. qRT-PCR was performed using standard protocols on an Applied Biosystem 7300HT Sequence Detection System. Briefly, 1.6 μl of a 1/10 dilution of cDNA in water was added to 10 μl of the 2×QuantiTect SYBR green PCR master mix, with 0.64 μl of each primer and water to yield 20 μl. The miRNA primers are listed in Table 1. PCR had an initial incubation step of 15 min at 95°C to activate the HotStarTaq DNA polymerase. Reactions were then amplified for 15 s at 95°C, 30 s at 58°C, and 34 s at 68°C for 40 cycles. A

Table 1 miRNA primers used in this study

Name	Sequence (5′-3′)
74	ATTTCGATTTTGGATTATTGGT
102	GAATTTGTTTCTTCATCCG
213	AAGGGATTGCTATTAGATTGA
367	TGTAGTCTGATTTTTCATGC
801	ATTGCTACTCAGTTTTGTTTTGA
QmiR-RT	GCGAGCACAGAAUUAAUACGACUC ACUAUAGG-d(U)18V(A,GorC) N(A,G,,C,or U)
QmiR-3′	GCGAGCACAGAAUUAAUACGAC

thermal denaturation protocol was performed at the end of the PCR to determine the number of products present in the reaction (Schmittgen and Zakrajsek 2000). All reactions were repeated in triplicate and "no template" controls were included for each sample. *P. cathayana* 5.8S ribosomal RNA (rRNA) was selected as the internal reference gene; 100 mmol L^{-1} NaCl for 72 h salt stressed and WT *P. cathayana* used the same qRT-PCR protocol.

Prediction of Potential Novel miRNA Targets

Computational predictions of putative targets for miRNAs have been performed more in plants than in animals, due to their almost perfect alignment with target genes (Rhoades et al. 2002). We used a miRNA target search tool (http://bioinfo3.noble.org/miRU2/) to predict novel targets and analyzed their functions using the web resources (http://www.popgenie.db.umu.se/popgenie/index.php) and (http://compbio.dfci.harvard.edu/index.html). The expression of miRNAs may induce responses to salt concentration. qRT-PCR was performed to verify the expression of two novel miRNAs.

Results

Sequencing of Small RNAs from *Populus*

Small RNAs were extracted from *P. cathayana* and polyadenylated with poly(A) polymerase, and a 50 nt RNA adapter was ligated to poly(A)-tailed RNA using T4 RNA ligase. RNAs were reverse transcribed and PCR was performed. The resultant PCR products were purified and inserted into the pMD19-T vector. Sequencing of the library and subsequent analysis was as described previously (see "Materials and Methods"). Sequencing of small RNAs generated 691 unique clones. Of these, 418 sequences (60%) from *P. cathayana* were complete, containing both 5′ and 3′ adapters. Forty-eight sequences matched noncoding RNAs including ribosomal rRNAs, tRNAs, snoRNAs, and snRNAs. Furthermore, miRNAs are approximately 18–24 nucleotides in length, so we used length as a screening criterion for sequences in the library. Thus, a total of 210 (54%) sequences in our library possibly represent full length mature miRNAs (data not shown).

Analysis of Potential *Populus* miRNAs

After masking of vector and adaptor sequences and removal of redundancy, the inserts between 18 and 24 nt in length were used in BLAST searches to identify candidate *P. cathayana* miRNAs. We screened the databases *Populus* DB and the *Populus* genome database. Among these, we identified 15 sequences with a perfect match and 80 with an almost perfect match (i.e., fewer than three mismatches) to genomic sequences.

The feature that best distinguishes plant miRNAs from other endogenous small RNAs is a candidate hairpin secondary structure. From the first discovery of miRNA-Lin4, all identified miRNAs have a typical miRNA secondary structure without exception. This is because the primary transcript (pri-miRNA) is transcribed by RNA polymerase II and contains an imperfect stem–loop secondary structure (Ambros et al. 2003a,b). All genomic sites for our cloned sequences, including 300 bp upstream and 20 to 30 bp downstream as well as the same length combination but in reverse order, and 200 bp upstream and 200 bp downstream were chosen for analysis by the m-fold program to identify secondary structure consistent with an miRNA. By these criteria we identified five candidate miRNAs, designated as ptc-miR801, ptc-miR74, ptc-miR102, ptc-miR367, and ptc-miR213 (Table 2). All precursor sequences for each miRNA obtained from the *P. cathayana* genome assembly form good hairpins, which have low dG value (Table 3).

Positive Detection of miRNAs

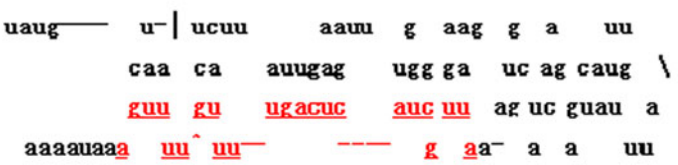
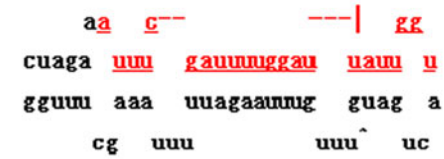
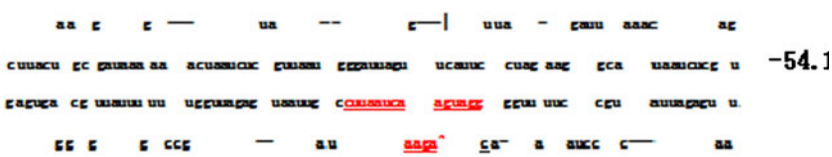
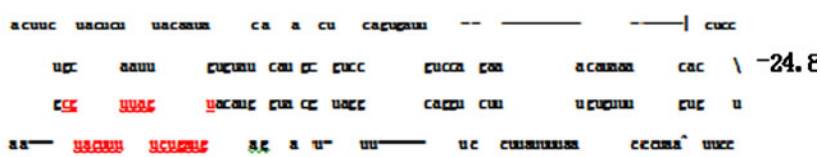
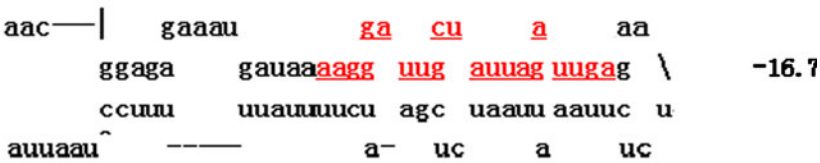
The expression of candidate miRNAs was examined by qRT-PCR analysis using total RNAs isolated from *P. cathayana*. We modified the poly(A)-tailed miRNA for use in a previously reported qRT-PCR assay (Fu et al. 2005) in order to generate a modified RT-PCR method for analyzing *P. cathayana* miRNA expression. A single sharp narrow peak for the dissociation curves was present in the SYBR Green real-time RT-PCR (Fig. 1). Each sample was tested three times and in triplicate reactions each time. Analysis of a single band of the appropriate size (about 80 bp) on a 4% agarose gel showed the specificity and sensitivity for detection of miRNAs.

Table 2 Summary of miRNA information

miRNA gene	sequence(5′-3′)	No. clones	Length (nt)	miRNA location
miR-801	AUUGCUACUCAGUUUUGUUUUGA	2	23	estExt_fgenesh4_pm.C_LG_II0615
miR-74	AUUUCGAUUUUGGAUUAUUGGU	1	22	estExt_Genewise1_v1.C_LG_X0562
miR-102	GAAUUUGUUUCUUCAUCCG	37	19	estExt_Genewise1_v1.C_34020003
miR-367	UGUAGUCUGAUUUUUCAUGC	1	20	eugene3.00111184
miR-213	AAGGGAUUGCUAUUAGAUUGA	1	21	estExt_fgenesh4_pg.C_LG_IV1453

nt nucleotides

Table 3 Hairpin structures of the precursors containing newly identified miRNA sequences (red) in *P. cathayan*

Name	Stem-loop structures of putative miRNA precursors	dG value
miR-801		-11.00
miR-74		-5.2
miR-102		-54.1
miR-367		-24.8
miR-213		-16.7

Underlined (red) bases represent the mature miRNA sequence

Underlined (red) bases represent the mature miRNA sequence

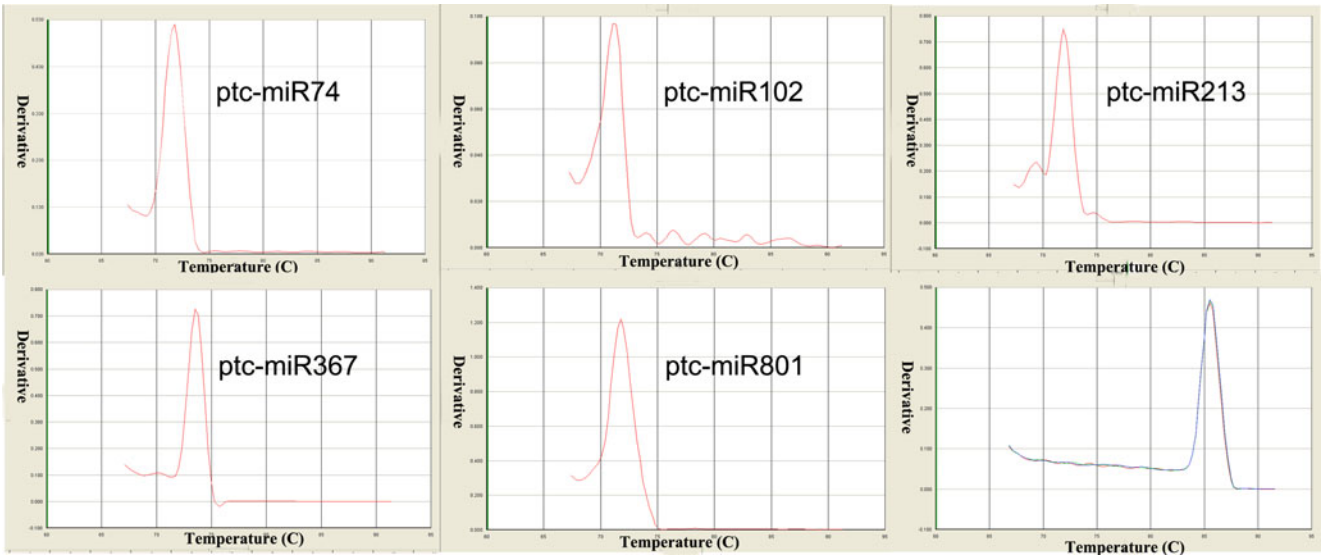


Fig. 1 Dissociation curves of *P. cathayana* candidate miRNAs. Dissociation curves of real-time PCR amplification of ptc-miR74, ptc-miR102, ptc-miR213, ptc-miR367, and ptc-miR801 and 5.8S rRNA

Expression Profile of *P. cathayana* miRNAs Across Different Organs

In order to predict possible roles for the novel *P. cathayana* miRNAs, we analyzed their expression levels in leaves, stems and roots by qRT-PCR analysis using primers with complementary sequences (Table 1). qRT-PCR detection represents a effective method for authenticating miRNAs (Lu et al. 2008). Ptc-miR801, ptc-miR74, ptc-miR367, and ptc-miR213 were detected in all tissues tested, with some exhibiting apparent tissue specificity (Fig. 2). The expression of ptc-miR801, ptc-miR74, ptc-miR367, and ptc-miR213 are robustly detected in leaf tissue. Ptc-miR367 was at best weakly expressed in stems and roots while all were highly expressed in leaves.

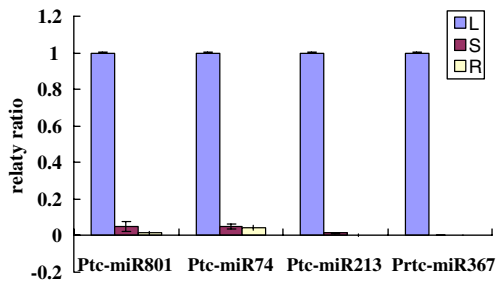


Fig. 2 Regulation of specific miRNAs by salt stress in 6-month-old *P. cathayana* treated with salt. Total RNA was extracted from salt-stressed plants after 72 h. The effect of salt treatment on the expression of ptc-miR801 and ptc-miR213 was analyzed by qRT-PCR. The 5.8S rRNA was selected as a reference

Prediction of Potential Novel miRNA Targets

Genes targeted by miRNAs in plants are thought to be regulated mainly via endonucleolytic cleavage of mRNAs, due to their near-perfect target complementarity, although recent studies indicate the existence of widespread translational inhibition (Brodersen et al. 2008). The targets of miRNAs are involved in the regulation of development and abiotic stress response. Using an miRNA target search tool, we identified potential targets for our novel miRNAs (numbers in parenthesis are mismatches). One such miRNAs (ptc-miR801) is predicted to target members of the NAC-domain protein (NAC) or Scarecrow-like (SCL) protein family which play an important role in salt stress. A candidate ptc-miR213 target is MYB4 (v-myb avian myeloblastosis oncogene ortholog), which may play an important role in plant stress tolerance. A ptc-miR74 appears to target genes belonging to the ubiquitin-associated family. A ptc-miR367 target is predicted to be PSBR (photosystem II subunit R; Table 4).

Analysis of Novel miRNAs Expression by qRT-PCR

We analyzed the expression of the ptc-miR801 and ptc-miR213 in plants treated for 72 h with salt (100 mM NaCl) as well as untreated plants. After salt exposure, RNA was extracted for qRT-PCR analysis. Compared with the non-stressed control, a significant upregulation of the two analysed miRNAs was revealed in response to salt stress (Fig. 3). Both genes were induced in response to salt stress.

Table 4 Potential targets for Ptc-miR801, Ptc-miR74, Ptc-miR213, and Ptc-miR367 in *P. cathayana*

MiRNA family	Predicted function	Target genes with number of mismatches in parentheses
Ptc-miR801	NAC-domain protein	LG_IV_13764001 (3)
	SCL	LG_V_15756001 (2.5)
Ptc-miR74	Auxin-responsive protein	LG_ IV _15312001 (3)
Ptc-miR213	UBA	
	estExt_fgenesh4_pg.C_LG_I2439 (3)	
	MYB	LG_IV_15789001 (1.5)
Ptc-miR367	PP2C-related	LG_III_14319001 (2.5)
	Protein kinase putative	LG_X_18480001 (2.5)
	PSBR	
	LG_XI_13632001(0),LG_XI_13635001(0)	
	Membrance protein	LG_VIII_7389001 (1)

All predicted miRNA targets and their miRNA displayed fewer than three mismatches (shown in parentheses)

Discussion

Recently, miRNAs have emerged as highly conserved endogenous non-coding small RNAs that exist extensively in plant organs and play critical regulatory roles in both transcriptional and post-transcriptional gene expression (Ambros 1989; Bartel 2004; Tong et al. 2007). It is therefore of critical interest to identify novel miRNAs from a wide spectrum of organisms and investigate gene regulation involving such miRNAs.

Since the identification of *lin-4* in *C. elegans* (Lee et al. 1993; Reinhart et al. 2000; Slack et al. 2000), hundreds of miRNAs have been identified in a wide range of organisms from plants to humans. To date there are 10,581 miRNA entries, distributed among 103 species, in the miRNA Registry Database (miRBase release 14.0), however thousands of miRNAs in various genomes still need to be identified (Ruvkun et al. 2004). The first plant miRNAs were found in *A. thaliana*, whose genome as a model plant was sequenced in 2000 (*Arabidopsis* Genome Initiative 2000). Subsequently, the draft genome sequence of *O. sativa*, as a monocotyledonous plant, was completed in 2002 (*O. sativa* Genome Initiative 2002). Furthermore, in September 2004, all the data concerning the sequence of the *Populus trichocarpa* genome has been published (*P. trichocarpa* Genome Initiative 2004). *Populus* is thus the third plant whose whole genome sequence have been described, and it is the first perennial woody species whose genome sequence has been determined. This represents the foundation of establishing an miRNA library for *Populus*. Moreover, there is little difference between *P. trichocarpa* and *P. cathayana*, both of which belong to the Tacamahaca group.

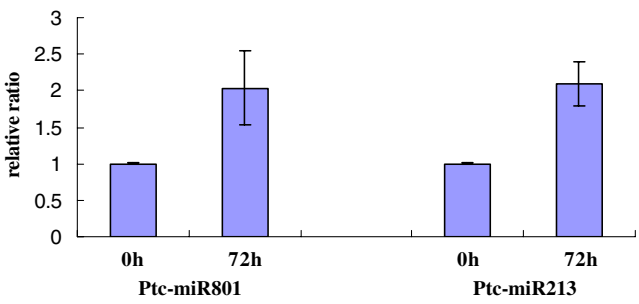


Fig. 3 Regulation of specific miRNAs by salt stress in one-and-a-half-month-old *P. cathayana* treated with salt. Total RNA was extracted from non-stressed control (0 h) and salt-stressed plants after 72 h. The effect of salt treatment on the expression of ptc-miR801 and ptc-miR213 was analyzed by qRT-PCR. The 5.8S rRNA was selected as a reference

Limitations of Constructing a Library

While constructing a library of small (18–24 nt) RNAs from *P. cathayana*, many short sequences were not able to be mapped to the genome of the plant, and there are several explanations for this result. Some sequences may be fragments of unidentified *Populus* miRNAs or undiscovered non-coding small RNAs such as siRNAs. One possibility is that the sequencing error rate is high, and there are many mistakes within the short sequence reads. The other possibility is that polymorphisms exist between different *Populus* individuals. The specimens we used in this study may be infected with pathogens but not presenting any symptoms, and thus their genomes may be contaminated.

Detection of Novel miRNAs

Identification of novel miRNAs can be achieved by several approaches. One method is to provide evidence of biogenesis characteristic of miRNAs, namely the identification of stem-loop structures that are characteristic for miRNAs. In the absence of evidence for biogenesis, experimental data such as qRT-PCR analysis can further verify a novel miRNA. Here, by sequencing a library of

small (18–26 nt) RNAs from *P. cathayana.*, we were able to add five novel miRNAs to the previously published 234 miRNA in *Populus.* We used qRT-PCR as a highly specific and sensitive detection tool, which requires only small amounts of plant material and is easily automated for high-throughput quantitation analysis, to validate the expression of novel miRNAs. A poly(A) tailed miRNA qRT-PCR assay was recently developed for miRNA detection, based upon an RT reaction initiated using a poly(A) tailed primer, and for most miRNAs the 5′ primer sequence is the same as the miRNA gene (Table 1) (Fu et al. 2005).

MiRNAs Regulate a Variety of Developmental and Physiological Processes

A variety of developmental and physiological processes are regulated by miRNAs. The majority of miRNAs are expressed in a developmental or organ-specific manner or both, which provides a few hints about their functions (Wightman et al. 1993). By performing qRT-PCR analysis of developmental expression for *Populus* miRNAs in order to predict roles for our novel Ptc-miRNAs (Table 3), we analyzed their expression levels in leaves, stems, and roots. This poly(A) tailed miRNA RT-PCR detection represents a useful criterion for miRNAs (Fu et al. 2005). Four novel ptc-miRNA families were detected in leaves, stems, and roots, with some exhibiting apparent tissue specificity. The expression levels of all transcripts are significantly higher in leaves than in stems or roots. One possibility is that miRNAs play critical regulatory roles in organ development, such as patterning of roots, stems, and leaves (Lai et al. 2003; Lewis et al. 2005; Zhang et al. 2006a, b). We speculate that the other reason we could not identify a miRNA that is upregulated in stems and roots is that all novel miRNAs were identified and confirmed in leaves tissues. However, we employed 6-month-old *P. cathayana* plantlets for extracting small RNA. The quantity of leaves at this age is more than stems and roots. It is possible that we have missed in our analysis miRNAs that are specific or abundant in stems and roots.

Targets of these Novel miRNAs

Further work aimed at identifying the target functions of these novel miRNAs will be needed to elucidate the roles of newly identified miRNAs in *P. cathayana*. For instance, miRNA targets may be involved in development and an abiotic stress response. Several transcription factors involved in development are targeted by miRNAs (Kidner and Martienssen 2005). Other miRNAs play a role in nutrient assimilation and responses to drought, low temperature, and other abiotic stresses (Sunkar and Zhu 2004). One of our novel miRNAs, ptc-miR801, is predicted to target NAC-domain protein members that may play an important role in salt stress. Recent studies have shown that *AtNAC2* gene expression was induced by salt stress in *Arabidopsis* (He et al. 2005). Ptc-miR801 likely targets the Scarecrow-like transcription factors, which belong to the GRAS protein family. GRAS transcription factors are a plant-specific protein family that includes GAI, RGA and SCR. Although they are plant-specific, only a few GRAS proteins have been characterized so far. However, it has been proven that GRAS proteins play regulatory roles in signal transduction, meristem maintenance, and development (Bolle 2004). Both NAC-domain protein and GRAS transcription factors may be ptc-miR801 family targets. Our predicted targets appear to be involved in diverse biological processes and functions. A candidate ptc-miR213 target is *MYB4*. *BcMYB1* was strongly induced by drought stress, and also responded to PEG, high salinity and low temperature to various extents (Chen et al. 2005). Other researchers showed that *AtMYB2* is a key regulatory factor for induction of the ADH1 (alcohol dehydrogenase 1) promoter by hypoxia (Hoeren et al. 1998). In our research, ptc-miR801 and ptc-miR213 were up-regulated in *P. cathayana* under salt stress for 72 h.

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