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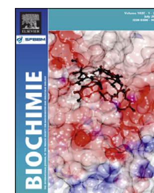

Research paper

Dynamic expression of novel and conserved microRNAs and their targets in diploid and tetraploid of *Paulownia tomentosa*

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ABSTRACT

MicroRNAs (miRNAs) play profound roles in plant growth and development by regulating gene expression. Tetraploid plants often have better physical characteristics and stress tolerance than their diploid progenitors, but the role of miRNAs in this superiority is unclear. *Paulownia tomentosa*, (Paulowniaceae) is attracting research attention in China because of its rapid development, wide distribution, and potential economic uses. To identify miRNAs at the transcriptional level in *P. tomentosa*, Illumina sequencing was used to sequence the libraries of diploid and tetraploid plants. Sequence analysis identified 37 conserved miRNAs belonging to 14 miRNA families and 14 novel miRNAs belonging to seven miRNA families. Among the miRNAs, 16 conserved miRNAs from 11 families and five novel miRNAs were differentially expressed in the tetraploid and diploid; most were more strongly expressed in the former. The miRNA target genes and their functions were identified and discussed. The results showed that several *P. tomentosa* miRNAs may play important roles in the improved traits seen in tetraploids. This study provides a foundation for understanding the regulatory mechanisms of miRNAs in tetraploid trees.

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1. Introduction

MicroRNAs (miRNAs) are a major type of endogenous 21–24 nucleotide (nt) single-stranded non-coding RNAs that are predominately derived from intergenic regions in both prokaryotes and eukaryotes [1–3]. They play important regulatory roles at the transcriptional and post-transcriptional levels during many growth and developmental processes, such as developmental timing, hormone responses, and reactions to environmental stress response [4–6]. MiRNAs were initially discovered in *Caenorhabditis elegans* [7].

In recent years, hundreds of small RNAs, especially miRNAs with low abundance, have been isolated in various higher plant species using next-generation sequencing technology and experimental and/or bioinformatical approaches, but most of these studies have focused on crop and annual plants; very few studies have involved forest trees [8–17]. For example, little information on miRNAs from *Paulownia* (Paulowniaceae) is available. *Paulownia tomentosa* is one of the most important indigenous fast-growing tree species in China, where it has a very wide distribution and is intercropped on 2.5 million ha of farmland. For effective breeding and improvement of *P. tomentosa*, autotetraploids were derived from diploid parent plants using colchicine [18]. Recently, the ecological characteristics and timber quality of the autotetraploid trees were found to be better than those of the corresponding diploids [19,20]. To clarify the underlying molecular mechanisms of prominent characteristics of the autotetraploid trees, we here adopted high-throughput sequencing technology to identify conserved and novel miRNAs of *P. tomentosa*. Differences in expression levels of these miRNAs between the diploids and autotetraploids were analyzed, and the potential roles of their target genes were investigated.

2. Materials and methods

2.1. Materials

Diploid (PT2) and autotetraploid (PT4) *P. tomentosa* were grown for 30 days in vitro on MS media containing 20 g L^{-1} sucrose, 1.0 mg L^{-1} NAA and 180 mg L^{-1}6-BA at 25 °C under a 16/8 h (light/dark) photoperiod, respectively. Leaves from plants were collected, frozen immediately in liquid nitrogen, and stored at −86 °C for total RNA extraction.

2.2. Small RNA library construction and sequencing

Total RNA was extracted from PT2 and PT4 leaves with Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the

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manufacturer's instructions. Two sRNA libraries were constructed and sequenced using the GAIIx platform. Briefly, 4 μg of total RNA were ligated with the 5′ and 3′ adapters successively. The reverse transcription reaction is used to create single stranded cDNA, and then amplified by 12 cycles PCR. The cDNA library were purified by polyacrylamide gel electrophoresis (PAGE) to select the fragments sized from 140 to 160 bp to produce the library for cluster generation and sequenced on the GAIIx platform were performed following the manufacturer's standard cBot and sequencing protocols.

2.3. Identification of miRNAs

The raw reads were produced with Illumina sequencing, and the low quality reads, adapters and contaminated reads were removed. The unique reads were then used to analyze the length distribution and mapped onto the *Paulownia* unigenes using miRDeep2. The perfectly matched reads were retained for further analysis. The reads matching non-coding RNA (including tRNA, rRNA, snoRNA, and other ncRNA, except microRNA) in non-coding RNA database [21] (Release 10) were deleted.

The remaining reads were searched against the plant mature microRNA of Sanger miRBase (Release 19.0) to identify the conserved miRNAs using the program Blastall, allowing two mismatches. The potential novel miRNAs were identified by using MIREAP and RNAfold [22] to fold flanking sequences and predict secondary structures. If the sRNA had a perfect stem loop structure and followed the other criteria described by Meyers et al., it was considered to be a novel miRNA [23].

2.4. Differential expression analysis of miRNAs in the PT2 and PT4

In the miRNA expression analysis, the abundance of miRNAs in the two libraries was normalized to one million, regardless of the total number of miRNAs in each sample. The fold change between the PT4 and PT2 was calculated as follows:

Fold change = miRNA normalized read counts in PT4 library/ miRNA normalized read counts in PT2 library.

The *P*-value was obtained according to the calculations as follows:

$$P(x|y) = \left(\frac{N_2}{N_1}\right) \frac{(x+y)!}{x!y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}}$$

$$C(y \le y\text{min}|x) = \sum_{y=0}^{y \le y\text{min}} p(y|x)$$

$$D(y \ge y\text{max}|x) = \sum_{y \ge y\text{max}}^{\infty} p(y|x)$$

$$\log 2 \text{ ratio} = \log 2(\text{fold change})$$

For direct comparison, *Paulownia* plantlets used to construct the libraries were grown under similar conditions.

2.5. Identification of miRNAs targets by degradome sequencing

To dissect miRNA-guided genes regulation in the PT2 and PT4 plants, two degradome libraries suitable for miRNA target identification were constructed following a previously described protocol [24,25]. In brief, poly(A) RNA was isolated and ligated to a 5′RNA adapter containing a MmeI recognition site. After reverse transcription using oligod(T) and PCR enrichment, the PCR products were purified and digested with *MmeI* (NEB, Ipswich, MA, USA). A double-stranded DNA adapter was then ligated to the digested products using T4 DNA ligase (NEB, Ipswich, MA, USA). The products were amplified using 20 PCR cycles and the final cDNA library was purified and sequenced on Illumina HiSeqTM 2000 system.

After initial processing, the unique sequence signatures were mapped to the database of *P. tomentosa* transcriptome sequences using SOAP software (http://soap.genomics.org.cn/) to define the coverage rate. The perfect matching sequences were retained and extend to 31 nt by adding approximately 15 nt of upstream of the sequence. All resulting reads (t-signature) were reverse-complemented and aligned to the miRNA identified in this study. Alignments with scores not exceeding 4 and having the 5′ end of the degradome sequence coincident with the tenth and eleventh nucleotides of complementarity to the sRNA were considered potential targets. Furthermore, t-plots were built according to the distribution of signatures (and abundances) along these transcripts. To better understand the functions of these targets, the Iprscan (http://www.ebi.ac.uk/tools/pfa/iprscan/) program was employed to gain the GO annotations and the pathway from the unigene database through Blastall hits against the available Pfam database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database in NCBI by an *E*-value threshold of less than 10^{-5}. The GO categorization results were expressed as three independent hierarchies for biological process, cellular component, and molecular function [26].

2.6. Quantitative real-time PCR

Identified *P. tomentosa* miRNAs and their target genes were experimentally validated using quantitative real-time PCR (qRT-PCR). For the experiment samples, diploid and tetraploid *P. tomentosa* tissue culture plants grown for 30 days, six months and one year were used. RNA from two biological replications were used for qRT-PCR, and total RNA was isolated using plant RNA extraction KIT (Aidlab Biotechnologies Co.,Ltd., Beijing, China). The stem-loop primers were designed for qRT-PCR as described previously [27]. The forward primers were designed based on the mature miRNA sequences and the reverse primer was the universal reverse primer, with U6 as the endogenous reference. The primers for target genes were designed with Beacon Designer, version 7.7 (Premier Biosoft International, Ltd., Palo Alto, CA, USA), and the 18S rRNA of Paulownia was chosen as an endogenous reference gene for normalization. All reactions were run in triplicate for each sample. The sequences of the primers are listed in Table S1. SuperScriptIII platinum SYBR Green one-step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA) and a CFX96 real time PCR system (Bio-Rad) were employed to detect and compare the expression levels. For each reaction, 500 ng of total RNA was mixed with 10 mL of SuperScriptIII platinum SYBR green PCR master mix and 4 pmol each of the reverse transcription, the forward and reverse primers in a final volume of 20 mL. The conditions for PCR amplification were as follows: 40 cycles at 95 °C for 15 s and 55 °C for 30 s. The $2^{-\Delta\Delta CT}$ relative quantization method was used to analyze relative changes in gene expression during the qRT-PCR experiments.

3. Results

3.1. Statistical analysis of sRNAs

The two sRNA libraries generated a total of 14,520,461 (PT2) and 13,109,201 (PT4) reads, respectively, by Illumina sequencing. After discarding the low-quality tags, adaptors, contaminants, sequences shorter than 18 nt, and sequences with poly-A tails, there remained

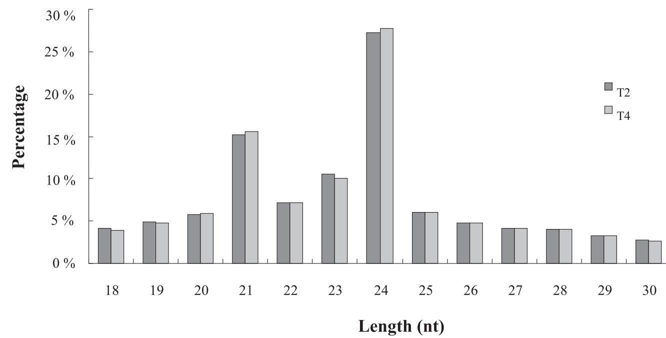

Fig. 1. Length distribution of sRNAs in diploid and tetraploid *Paulownia tomentosa*.

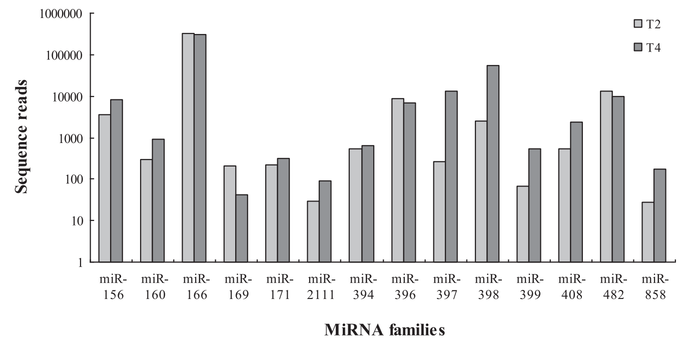

Fig. 2. MiRNA families of *Paulownia tomentosa* sequence reads.

8,135,669 (PT2) and 11,919,705 (PT4) clean reads for further analysis. The majority of the clean reads were 21–24 nt in length in both libraries, typical of Dicer-derived products (Fig. 1). The most abundant class of sRNAs was 24 nt, approximately 27% on average, while the 21-nt sRNAs represented approximately 15% of the clean reads, on average. These results were similar to the sRNAs of many plant species, including *Arabidopsis*. A total of 45.14% of the sRNAs matched the *Paulownia* unigenes in the two libraries, and the matched clean tags were classified and annotated into miRNAs, snoRNAs, snRNAs, and tRNAs using the GenBank, Rfam, and miRBase databases (Table 1). The same amount of RNA was used to construct these two libraries and the samples were prepared in a similar manner. The counts of sRNAs in the two libraries were very similar (Table 1), indicating that chromosome doubling had little effect on the classification of *P. tomentosa* sRNAs. Those sRNAs that could not be annotated to any category were further analyzed to predict novel miRNAs and new conserved miRNAs.

3.2. Identification of conserved and novel miRNAs

The miRNA sequences were evaluated using miRBase 18.0. Those that differed from known miRNAs by two mismatches or fewer were defined as conserved miRNAs. A total of 35 (PT2) and 37 (PT4) distinct conserved miRNAs and miRNAs* (miRNA star) sequences belonging to 14 miRNA families were identified in the two libraries (Table S2). Among these families, pau-miR166 was the most abundant, representing approximately 91.55% of the reads generated from the PT2 library, while pau-miR858 and pau-miR2111 were the least abundant (Fig. 2). Twenty-one miRNAs had more than one hairpin structure, indicating that their pri-miRNAs were different and that these miRNAs families had more members than others.

In addition to conserved miRNAs, the remaining unannotated sRNAs yielded 13 (PT2) and 14 (PT4) sequences belonging to seven families that were predicted as potential novel miRNAs using updated plant miRNA annotation criteria [23]. Their sequence information and hairpin structures are given in Table S3 and Fig. S1.

3.3. Expression analysis of miRNAs in diploid and tetraploid *P. tomentosa*

The expression levels of miRNAs as indicated by the relative abundances of their sequences were compared between PT2 and PT4. All conserved and novel miRNAs were normalized and analyzed by calculating their fold-change ratios and *P*-value. MiRNAs with *P*-values less than 0.05 and log 2 ratio <-1 or >1 were considered to have significantly-different expression levels. Sixteen conserved miRNAs belonging to 11 miRNA families were significantly differently expressed between PT2 and PT4 (Fig. 3A). Among them, miRNA pau-miR166-3p-1, pau-miR169 and pau-miR169-3p were expressed more weakly in PT4 than in PT2, while the others were expressed more strongly; pau-miR169-3p had the lowest relative expression and pau-miR397-3p the highest. We also detected five novel miRNAs with significantly different expression levels (Fig. 3B), all of which were expressed more strongly in PT4. Among all of the novel miRNAs, only pau-miR4, pau-miR4-3p, and pau-miR5 had lower relative expressions in PT4. Of the novel miRNAs, pau-miR2 had the highest relative expression in PT4 and pau-miR4-3p the lowest.

3.4. Target identification for Paulownia miRNAs by degradome analysis

To better understand the functions of the identified *P. tomentosa* miRNAs in this study, a recently developed degradome sequencing approach [24,25] was applied to identify the targets of the *P. tomentosa* miRNAs. A total of 20,991,041 (PT2) and 19,870,270 (PT4) raw reads of 3′ cleavage fragments were generated. After removal of low quality sequences, adapter sequences and redundancy reads, 7,256,739 and 6,522,175 unique reads from PT2 and PT4 degradome libraries, respectively, could be perfectly mapped to the *paulownia* transcriptome (Table 2). By PAIRFINDER software analyzed, 221 miRNA-targeted transcript pairs were confirmed by degradome sequencing (Table S4). Based on the signature relative abundance at the target sites, these target transcripts were selected

Table 1
Categories and statistical summary of sRNAs in diploid and tetraploid *Paulownia tomentosa*.

Class	PT2				PT4			
	Unique	%	Reads	%	Unique	%	Reads	%
miRNA	2763	0.10	960,773	8.29	2429	0.10	911,671	8.84
snoRNA	1216	0.04	13,450	0.12	1272	0.05	17,407	0.17
snRNA	1486	0.05	4041	0.03	1521	0.06	4315	0.04
tRNA	21,508	0.77	349,773	3.02	14,182	0.59	250,937	2.43
Other	59,287	2.13	1,041,270	8.98	46,299	1.92	926,515	8.99
unannotated	2,698,818	96.90	9,222,920	79.56	2,348,476	97.28	8,200,200	79.53

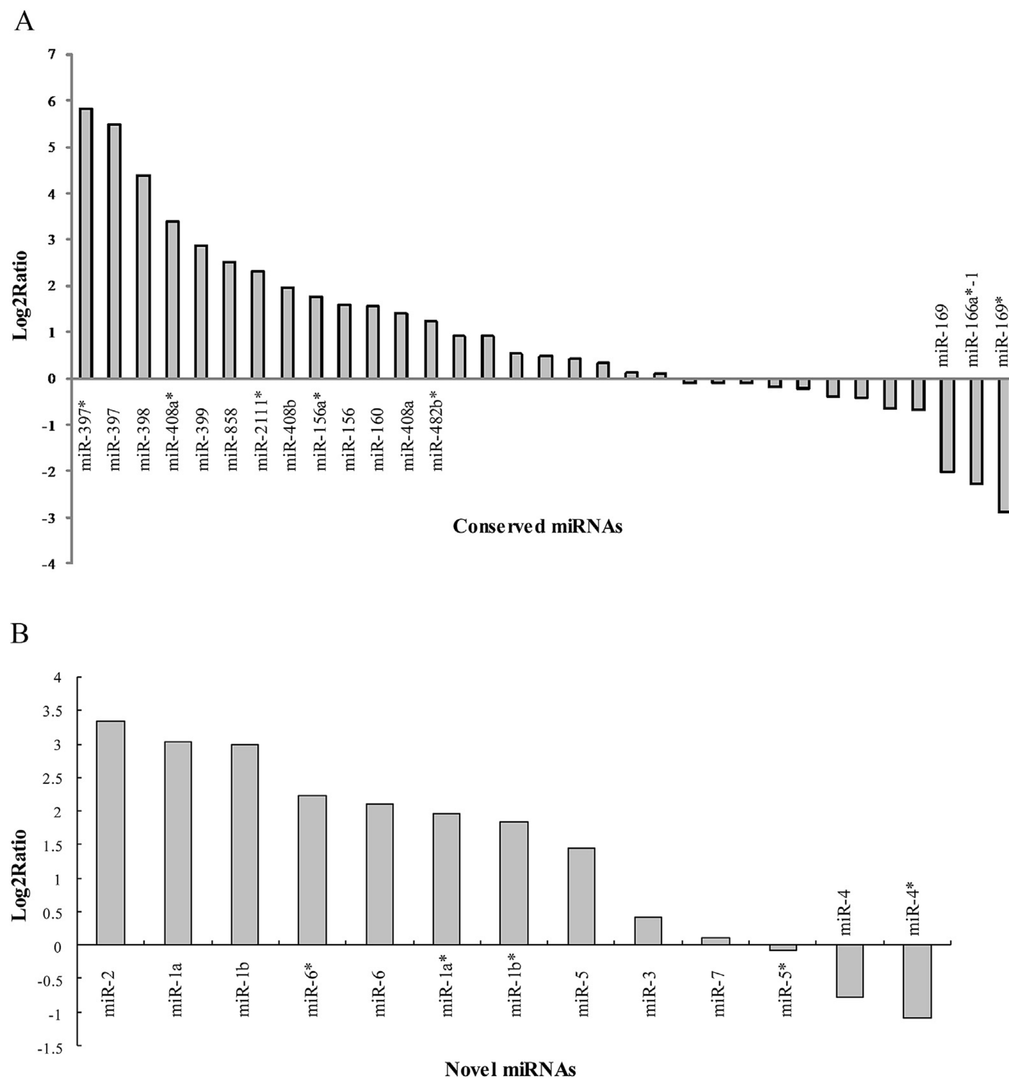

Fig. 3. Differential expression of miRNAs in diploid and tetraploid *Paulownia tomentosa*. A. Differential expression of conserved miRNAs. B. Differential expression of novel miRNAs.

and categorized as I, II or III (Table S4, Fig. 4) as previously described [24,25]. Among these identified targets, 130 targets (130 cleavage sites) belonged to category I, 102 targets (195 cleavage sites) were found to belong to category II, and only 22 (30 cleavage sites) were in category III (Table S4).

A BlastX search of the SwissProt database showed that these miRNA targets shared homology with other plant proteins (Table S4). The pathways of these target genes were then analyzed using the KEGG database and GO analysis (Table S4, Fig. 5). The predicted genes were involved in a broad spectrum of cell developmental processes, including energy metabolism, signal transduction, and transcriptional regulation, and have important functions during plant growth. For example, pau-miR398, which was more strongly expressed in PT4, targets the gene encoding serine/threonine-protein kinase abkC, a kinase enzyme that phosphorylates the OH group of serine or threonine. Serine/threonine kinase receptors play a role in regulating cell proliferation, programmed cell death (apoptosis), cell differentiation, and embryonic development [28]. Genes encoding myb-related protein, transcription repressor MYB5, and transcription factor WER (MYB66) are targeted by pau-miR858. MYB is a transcription factor that is involved in plant growth and abiotic stress resistance. Members of these proteins families regulate gene expression in response to salt, drought, and cold stresses [29]. The pau-miR169, which was more weakly expressed in PT4, was predicted to target the gene encoding nuclear transcription factor Y subunit (NFYB). NFYB has been shown to interact with CCAAT/enhancer binding protein zeta, CNTN2, TATA binding protein and MYC [30].

Table 2
Summary of reads produced by degradome sequencing in PT2 and PT4.

Types of reads	Number of reads	
	PT2	PT4
Total reads	20,991,041	19,870,270
High quality	20,959,687	19,823,683
Adaptor 3 null	4895	5084
Insert null	12	22
Adaptor 5 contaminants	67,501	61,085
Smaller than 18 nt	7438	6242
Clean reads (unique)	20,879,841 (9,399,611)	19,751,250 (8,525,686)
Mapping to transcriptome reads (unique)	17,147,888 (7,256,739)	16,347,595 (6,522,175)

3.5. Expression pattern analysis of P. tomentosa miRNAs and their targets

To confirm the expression of identified miRNAs and detect their dynamic expression in diploid and tetraploid plantlets at different

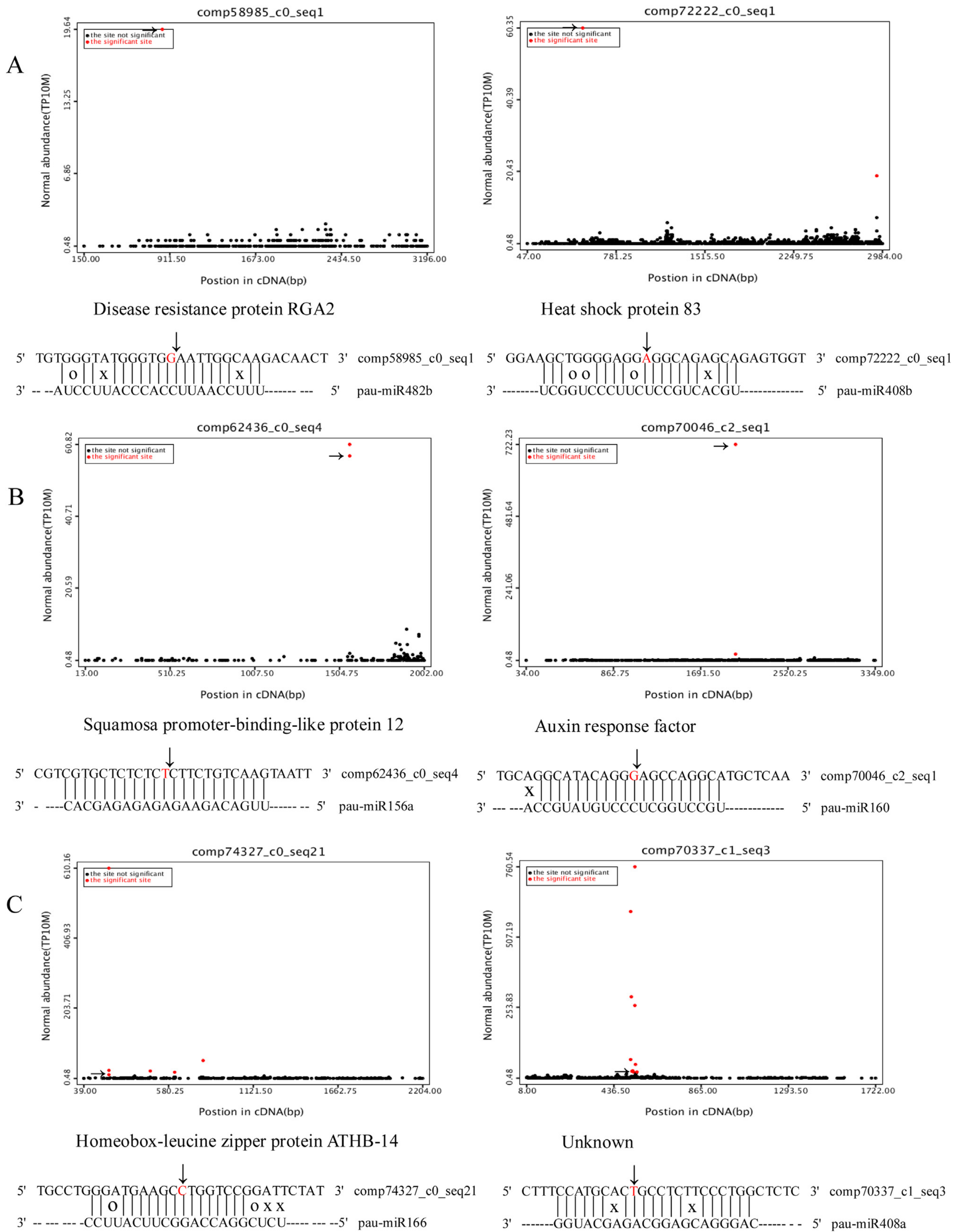

Fig. 4. Target plots (t-plots) of miRNA targets in different categories confirmed by degradome sequencing. (A) t-plot (top) and miRNA: mRNA alignments (bottom) for two category I targets, comp58985_c0_seq1 and comp72222_c0_seq1 transcripts. The arrow indicates signatures consistent with miRNA-directed cleavage. The solid lines and dot in miRNA: mRNA alignments indicate matched RNA base pairs and GU mismatch, respectively, and the red letter indicates the cleavage site. (B) comp62436_c0_seq4 and comp70046_c2_seq1, a category II target for pau-miR156a and pau-miR160. (C) comp74327_c0_seq21 and comp70337_c1_seq3, a category III target for pau-miR166 and pau-miR408a.

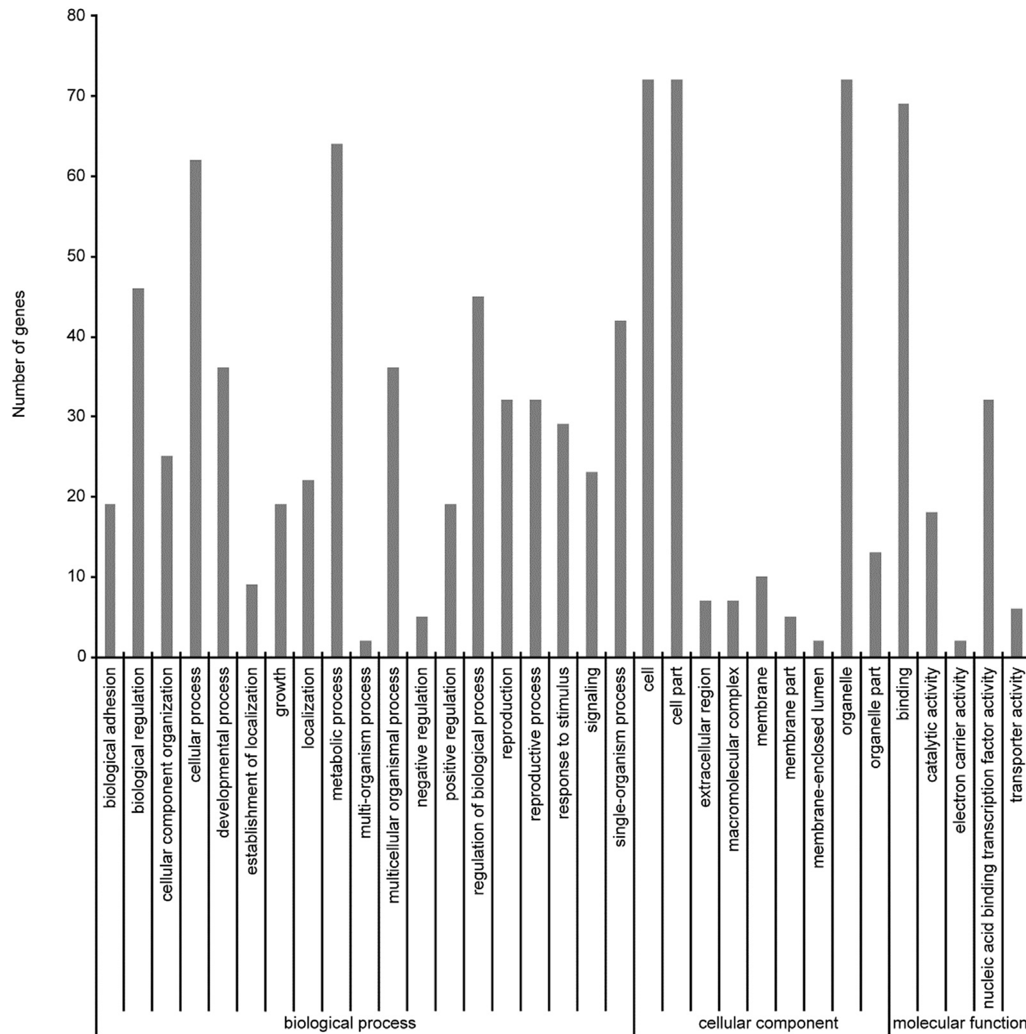

Fig. 5. Gene Ontology analysis of conserved miRNA targets in *Paulownia tomentosa*.

treatment stages, the expression of twelve conserved and two non-conserved miRNAs with significantly altered sequencing counts following sequencing were analyzed by qRT-PCR. The RNA not only included 30 days growth diploid and tetraploid plantlets, but also six months and one year plants. As shown in Fig. 6, miRNA expression levels varied over the course of the plant growth. From the results, the expression of nine miRNAs (pau-miR156a, pau-miR166-3p, pau-miR169-3p, pau-miR2111-3p, pau-miR396b, pau-miR408b, pau-miR858, pau-miR6, and pau-miR6-3p) in diploid and tetraploid increased at early stage and then decreased in the late stage, following the same mode with the growth of plantlets. The expression of pau-miR397 and pau-miR482b were decreased at the six months and one year plants stages, and pau-miR169, pau-miR398 and pau-miR408a-3p have the different expression mode. Also, we found that six months of *P. tomentosa* plantlets has the most abundant expression of miRNA, which probably means this period was the most important time of plant growth. In the comparison of miRNA expression at different growth stages of diploid and tetraploid, we have got two biological replicates results that have been consistent. The expressions of most miRNAs showed similar trends to those determined by Solexa sequencing only a few different. But only seven miRNAs in the three stages have the same expression trend between diploid and tetraploid. The expressions of pau-miR169-3p, pau-miR396b and pau-miR482b in three stages of tetraploid were all less than the diploid, pau-miR397, pau-miR398 and pau-miR408a-3p were on the contrary. The expressions of the rest of miRNAs in three stages of tetraploid were significantly different from its diploid. These results indicated that the expression of miRNAs were very complex and varied during the growth and development of *P. tomentosa*.

To test the potential correlation between miRNAs and their target genes, the expression patterns of eleven miRNA targets at different development stages by using real-time PCR were analyzed. These targets included squamosa promoter-binding-like protein 12 (comp62436_c0_seq4) targeted by pau-miR156a, Cysteine proteinase RD21a (comp68533_c0_seq1) and Mitochondrial import receptor subunit TOM6 homolog (comp41425_c0_seq1) targeted by pau-miR396b, Laccase-4 (comp13315_c0_seq1) and ABC transporter G family member 7 (comp66172_c0_seq2) targeted by pau-miR397, Disease resistance protein RPP13 (comp75351_c0_seq5) and Disease resistance protein RGA2 (comp58985_c0_seq1) targeted by pau-miR482b; Pentatricopeptide repeat-containing protein At3g09060 (comp66232_c1_seq3) and Heat shock protein (comp72222_c0_seq1) targeted by pau-miR408b; Transcription factor WER (comp31199_c0_seq2) and Myb-related protein P (comp56068_c0_seq1) targeted by pau-miR858. As expected, the expression levels of the most genes were inversely correlated with these of the corresponding miRNAs

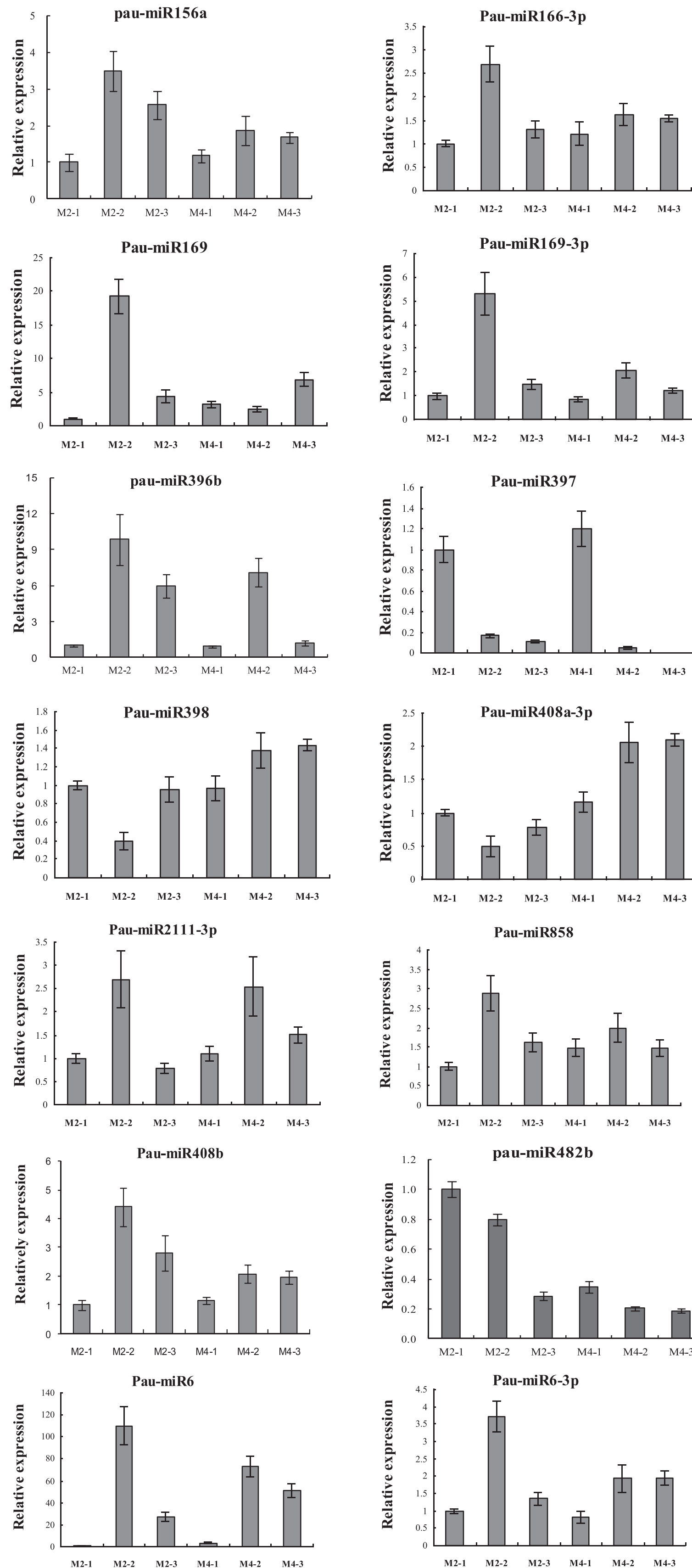

Fig. 6. Results from qRT-PCR of miRNAs in *P. tomentosa*. RNAs were isolated from diploid and tetraploid plantlets grown in 30 days (M2-1, M4-1), 6 months (M2-2, M4-2), one year (M2-3, M4-3). The expression levels of miRNAs were normalized to U6. The normalized miRNA levels in the M2-1 were arbitrarily set to 1.

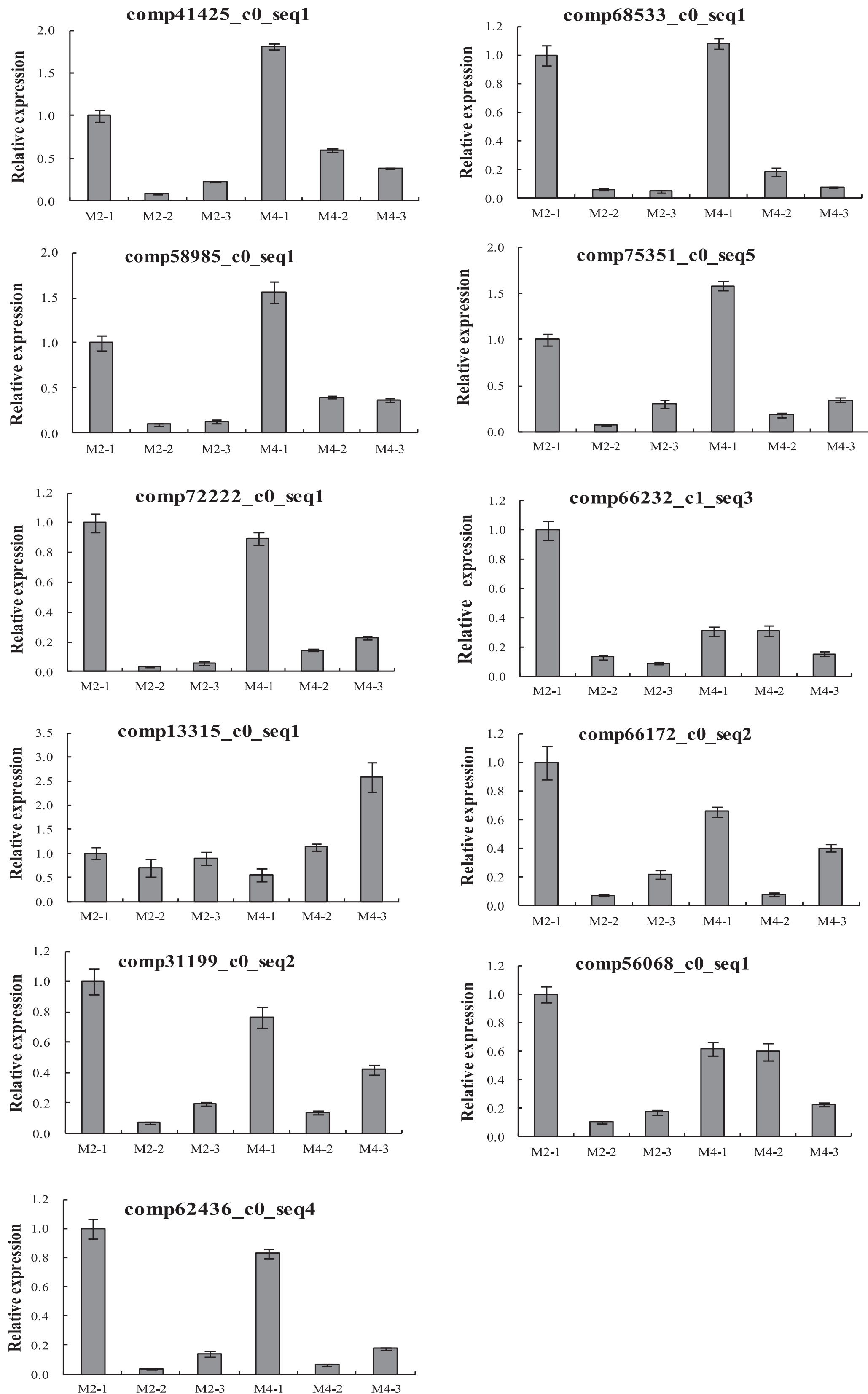

Fig. 7. Relative expression levels of the target genes in *P. tomentosa*. RNAs were isolated from diploid and tetraploid plantlets grown in 30 days (M2-1, M4-1), 6 months (M2-2, M4-2), one year (M2-3, M4-3). The expression levels of targets were normalized to 18S rRNA. comp68533_c0_seq1 (cysteine proteinase RD21a) and comp41425_c0_seq1 (mitochondrial import receptor subunit TOM6 homolog) targeted by pau-miR396b; comp13315_c0_seq1 (Laccase-4) and comp66172_c0_seq2 (ABC transporter G family member 7) targeted by pau-miR397; comp75351_c0_seq5 (disease resistance protein RPP13) and comp58985_c0_seq1 (disease resistance protein RGA2) targeted by pau-miR482b; comp66232_c1_seq3 (pentatricopeptide repeat-containing protein At3g09060) and comp72222_c0_seq1 (heat shock protein) targeted by pau-miR408b; comp31199_c0_seq2 (Transcription factor WER) and comp56068_c0_seq1 (Myb-related protein P) targeted by pau-miR858; comp62436_c0_seq4 (squamosa promoter-binding-like protein 12) targeted by pau-miR156a.

except the targets of Laccase-4 and ABC transporter G family member 7 (Fig. 7). During the three developmental stages, pau-miR156a, pau-miR408b and pau-miR858 expressed at a relatively lower level in the PT4 than in the PT2 at the six months and one year plants stages, while its target gene encoding the Squamosa promoter-binding-like protein 12, Pentatricopeptide repeat-containing protein, Heat shock protein, Transcription factor WER and Myb-related protein P expressed in the reverse way (Fig. 7). Moreover, a reverse trend was noted between pau-miR396b and its target genes coding for disease resistance protein RPP13 and disease resistance protein RGA2, and between pau-miR482b and its target gene coding for cysteine proteinase RD21a and mitochondrial import receptor subunit TOM6 in the PT4 as compared to the PT2. The expression levels of pau-miR396b and pau-miR482b in PT4 were significantly lower than in the PT2 at all treatment stages, while the reverse was true for their target genes (Fig. 7). These results suggested that the differentially expressed miRNAs caused different expression levels in their target genes at different development stages. Furthermore, the inverse correlation between miRNAs and their target genes were confirmed.

4. Discussion

The *P. tomentosa* tetraploid has as the same genotype as the diploid, except that the genome doubled. In this study, we compared the sRNAs in diploid and tetraploid *P. tomentosa* by deep sequencing against the *P. tomentosa* transcriptomes. sRNAs in both libraries were identified and classified. The most abundant sRNA sequence length was 24 nt, followed by 21 nt, within the range of miRNAs. We identified 37 unique conserved miRNA sequences and 14 novel miRNAs with significantly different expression levels between PT2 and PT4 and predicted their targets and pathways.

According to the GO analysis of conserved miRNAs targets in *P. tomentosa*, the target genes were classified into three functional categories: cellular component, molecular function, and biological process. Each category included several subcategories and indicated that the target genes of *P. tomentosa* conserved miRNAs were predicted to have significant functions in cell structure, metabolism, signal transduction, response to stimuli, and other areas. The peak of gene distribution was in molecular building function, while cell, cell part, and metabolic process were also very important; more than 70 genes played roles in these areas (Fig. 5). These miRNAs may be closely related to the cell structure differences between the tetraploid and diploid plants [19].

As we know, the miRNAs are processed by Dicer-like proteins from stem–loop regions of longer RNA precursors called pri-miRNAs. In theory, the tetraploids, which have doubled genomes, should have more pri-miRNAs. By comparing the miRNA expression levels between these two libraries, we found that the doubling of the *P. tomentosa* genome did not simply increase the expression of miRNAs two fold. Some miRNAs were expressed at similar levels in PT4 and PT2, while others were expressed much more strongly or more weakly in PT4 than in PT2. These data suggested that the regulated function of miRNAs depended on the plant metabolic system, and the miRNA products varied with how many pri-mRNAs were processed by Dicer-like enzymes, affecting plant growth [31]. All these findings indicated that miRNA regulation may be more complex in tetraploids than in diploids, resulting in better physiological and biochemical traits.

The miRNAs of the pau-miR397 family, which showed the highest expression levels in PT4 relative to PT2, were identified to target genes of ABC transporter G family member 7, Laccase-4, and Laccase-11 (Table S4). Similarly, in *Arabidopsis*, miR397a probably targeted members of the laccase family, whereas another family member, miR397b, displayed better complementarity with another casein kinase gene. The fact that miR397 can target laccase provides a unique tool to investigate the function of laccase in higher plants [32]. Moreover, we have also identified the target of pau-miR398, studies in other plants suggest that its target and function were probably the same as those of miR397. The miRNA Pau-miR394 targeted gene of a F-box protein. F-box family proteins are critical determinants for controlling Skp1-Cullin-F box complex substrate selection and are key regulators in many cell-signaling, transcription, and cell-cycle pathways [33]. MiR408 was highly conserved in rice, *Arabidopsis*, and *Populus*, while *P. tomentosa* had at least four family members (pau-miR408a, pau-miR408a-3p, pau-miR408b, and pau-miR408b-3p); all were more strongly expressed in the PT4 library. In *Arabidopsis*, miR408 may target mRNAs encoding plantacyanins and peptide chain release factor [32].

Wheat tetraploids have a high level of salinity tolerance and, according to previous research, the expressions of miR397, miR398, miR399, and miR408 were induced in response to drought, cold, abscisic acid, oxidative, and salt stresses [34–36]. A similar situation occurred in *P. tomentosa*. In addition, pau-miR858 was significantly more strongly expressed in the PT4 library; it targets transcription factor MYB and is widely involved in the genetic information processing and transcription response to abiotic stress pathways. The targets of Pau-miR408b are pentatricopeptide repeat proteins, whose genes share certain features with disease resistance genes, and their “nomadic” character suggests that their evolutionary expansion in plants may have involved novel molecular processes and selective pressures [37]. This finding indicated that the *P. tomentosa* tetraploid may have better stress tolerance than the diploid.

Overall, the significantly altered expression of miRNAs in the *P. tomentosa* tetraploid may play important roles in regulating target genes to result in improved biological characteristics and timber quality relative to the diploid.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2014.02.008.

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