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Original Article

# Identification of microRNA and analysis of target genes in *Panax ginseng*

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ABSTRACT

*Objective:* Ginsenosides, polysaccharides and phenols, the main active ingredients in *Panax ginseng*, are not different significantly in content between 3 and 5 years old of ginsengs called Yuan ginseng and more than ten years old ones called Shizhu ginseng. The responsible chemical compounds cannot fully explain difference in efficacy between them. According to reports in *Lonicerae Japonicae Flos* (Jinyinhua in Chinese) and *Glycyrrhizae Radix* et *Rhizoma* (Gancao in Chinese), microRNA may play a role in efficacy, so we identified microRNAs in *P. ginseng* at the different growth years and analyzed their target genes.
*Methods:* Using high-throughput sequencing, the RNA-Seq, small RNA-Seq and degradome databases of *P. ginseng* were constructed. The differentially expressed microRNAs was identified by qRT-PCR.
*Results:* A total of 63,875 unigenes and 24,154,579 small RNA clean reads were obtained from the roots of *P. ginseng*. From these small RNAs, 71 miRNA families were identified by bioinformatics target prediction software, including 34 conserved miRNAs, 37 non-conserved miRNA families, as well as 179 target genes of 17 known miRNAs. Through degradome sequencing and computation, we finally verified 13 targets of eight miRNAs involved in transcription, energy metabolism, biological stress and disease resistance, suggesting the significance of miRNAs in the development of *P. ginseng*. Consistently, major miRNA targets exhibited tissue specificity and complexity in expression patterns.
*Conclusion:* Differential expression microRNAs were found in different growth years of ginsengs (Shizhu ginseng and Yuan ginseng), and the regulatory roles and functional annotations of miRNA targets in *P. ginseng* need further investigation.



## 1. Introduction

Roots of *Panax ginseng* C. A. Meyer, play important roles in modulating the immune system as well as combating stress, cancer and diabetes in human beings (Chen, Li, Qiu, Qiu, & Qu, 2018; Li et al., 2022; Shin et al., 2018; Sun et al., 2014), in which ginsenosides, polysaccharides and phenols are the main responcialbe components. Yuan ginseng and Shizhu ginseng are cultivated ginsengs at different growth years. Generally, Yuan ginseng can be used as medicine for about 3–5 years, and it takes more than 10 years for Shizhu ginseng to be used as medicine. In clinical practice of traditional Chinese medicine, they are different in efficiency though they are same in contents of the major components. As a perennial tetraploid plant species, P. ginseng has a large genome with a considerable number of expressed sequence tags (ESTs) that have been sequenced (Chen et al., 2011; Choi et al., 2005; Sathiyamoorthy, In, Gayathri, Kim, & Yang, 2010), and the entire genome of P. ginseng has been published in 2017 (Xu et al., 2017). The transcribed genomic data of P. ginseng have been established. Ginsenosides cannot fully explain the great difference in efficacy between Yuan ginseng and Shizhu ginseng. Considering that small biomolecules may play a role in clinical efficacy, small RNA provides substantial information and new opportunities for further understanding the gene functions in *P. ginseng*.

MicroRNAs (miRNA) are a class of small endogenous, single-chain, non-coding RNAs with the lengths of about 18–21 nt. Accumulating evidence has demonstrated that miRNAs play key roles in gene expression regulation by modulating cell differentiation, proliferation and apoptosis through direct cleavage of transcripts or translational repression at the post-transcriptional level (D'Ario, Griffiths-Jones, & Kim, 2017), thus contributing to the new ways and intervention targets for disease treatment. miRNA in *P. ginseng*

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may also have a certain pharmacological regulatory effect and exert clinical efficacy.

The regulatory roles of miRNAs in plant growth, development, as well as biotic and abiotic stresses have been revealed. For example, miR393 targets growth hormone receptor TIR1, AFB2orF-Boxto control the distribution of auxinin plants (Vidal et al., 2010); miR172 regulates the development of flowers and their organs by targeting APETALA2 (AP2) and inhibiting AP2 expression in Arabidopsis (Shivaraj, Jain, & Singh, 2018). It is found that food-derived exogenous plant Mir-168a can enter the circulation and various organs through the mouse gastrointestinal pathway, and regulate mouse LDLRAP1 protein expression across species in the liver. This study showed that exogenous plant miRNAs in food can regulate the expression of mammalian target genes across borders for the first time (Zhang et al., 2018). Zhou et al. (2015) studied *Lonicerae Japonicae Flos* (Jinyinhua in Chinese), a commonly used traditional Chinese medicine for influenza treatment. MIR2911 can still be completely preserved in the decoction of *Lonicerae Japonicae Flos*. *In vitro* cell experiments showed that MIR2911 can target and inhibit multiple viral genes of influenza A virus. Mice experiments showed that MIR2911 in the blood and lungs of mice fed with *Lonicerae Japonicae Flos* decoction significantly increased. MIR2911 can inhibit the replication of various influenza A viruses in mice, reduce the weight loss caused by virus infection mortality rate. It is suggested that plant miRNA may also be one of the active components and effective substances of traditional Chinese medicine. Clinical trials have confirmed that the miRNA in *Lonicerae Japonicae Flos* decoction can be effectively absorbed by the human body through drinking, and can effectively inhibit the replication of the new coronavirus in the body (Zhou et al., 2020). As target gene prediction and validation are keys to the biological functional annotation of miRNAs in plants, we herein not only constructed the small RNA library, but also predicted and validated the target genes of the semiRNAs by bioinformatics analysis and degradome sequencing, finally identifying 233 known and novel miRNAs from the small RNA dataset. Degradome sequencing, as a high-throughput experimental method for identifying the target transcripts of plant miRNAs (Wu, Wang, Ma, Yuan, & Lu, 2012), can be employed to verify the miRNA target genes in plants accurately on a large scale (Xu et al., 2012; Mathiyalagan et al., 2013). Using this technology, we identified 13 target genes of eight known and novel miRNAs in *P. ginseng* in this study. The homologue to target gene possibly allows a miRNA to have multiple degradation sites, so a target gene with the same or similar conserved structure can be targeted by the same miRNA (Liu et al., 2014). Consistently, the target genes of *P. ginseng* miRNAs were associated with energy metabolism, biological stress, disease resistance and immunity based on the annotated functions related to transcription factor, F-Box, auxin responsive factors, DNA binding proteins, Dicer enzyme and $Ca^{2+}$-ATP enzyme inhibitors.

## 2. Materials and methods

### 2.1. Plant material and RNA extraction

The fresh roots of 15-year Shizhu ginseng and 6-year Yuan ginseng purchased from Fusong (Jilin Province, China) were treated in accordance with the following procedures. Clean *P. ginseng* washed with ultra-pure water was sterilized in 70% ethanol and then rinsed using sterile water. After air-drying, it was cut into pieces, followed by quick-freezing with liquid nitrogen for total RNA extraction using Trizol reagent (Qiagen, Dusseldorf, Germany) according to the manufacturer's protocols for *P. ginseng* root RNA extraction. Following the purification procedure with DNase I, the extracted total RNA was determined for quality and integrity by agarose gel electrophoresis (Liuyi, Beijing, China), TGem micro spectrophotometer (Tiangen, Beijing, China) and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, USA).

Roots of three plants of Yuan ginseng with the same growth environment and the same growth period were taken to repeat the above operation. After passing the quality test (RNA integrity number (RIN) >7.0, 28S/18S $\geq$ 0.7), the three tubes were mixed and used for sequencing. The operation of roots of Shizhu ginseng was the same as above.

### 2.2. High-throughput sequencing

After quality inspection, total RNA was used to perform deep sequencing as per the following procedures: transcriptome sequencing, small RNA sequencing and degradome sequencing. Construction and sequencing of transcriptome and small RNA library were performed using Illumina HiSeq™ 2500 (Illumina, San Diego, USA), consuming no <10 μg of total RNA from Yuan ginseng and Shizhu ginseng. The basic reads were converted into raw data by base calling. Low-quality reads were filtered, and the reads with 5′ primer contaminants and poly (A) were removed. The reads without 3′ adapter and insert tag, the reads shorter than 18 nt and longer than 41 nt from the raw data were filtered, and the clean reads were obtained. Degradome sequencing was conducted for the known miRNA reads from small RNA sequencing as well as the unigenes from transcriptome sequencing (Fang et al., 2013).

### 2.3. Differential expression analysis

The expression levels of miRNAs in Yuan ginseng and Shizhu ginseng were normalized and calculated in reads per kb per million read values during assembly and clustering. Significances of differential miRNA expressions between the two *P. ginseng* species were determined by *P*-value $\leq$ 0.05 and fold change (FC > 2 or FC < 0.5) with the random test, and corrected using the false discovery rate (FDR).

### 2.4. Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from *P. ginseng* with Trizol reagent according to the manufacturer's protocol. Real-time PCR was conducted on the basis of a two-step reaction using QuantiFast® SYBR® Green PCR (Perfect Real-Time) kit (Qiagen, Dusseldorf, Germany) on LightCycler® 480 IIsystem (Roche, Basel, Swiss), with U6 as the internal reference gene.

## 3. Results

### 3.1. Analysis results of transcriptome sequencing

Ginsenosides were distributed in many tissues in *P. ginseng*, including berry and leaves, especially in root tissue with high contents. To identify the differences between the clinical effects of *P. ginseng* species, we sequenced mRNA samples from two cultivors of *P. ginseng* (Yuan ginseng and Shizhu ginseng) using Illumina Hiseq™ 2500 system. Approximately 52.4 million raw reads and ~65 billion base pairs (bp) were ultimately obtained. In order to acquire high-quality reads, those with low quality were filtered out by setting the quality threshold at 20, and the length threshold was defined as 70% to remove the reads shorter than 35 bp. The high-quality reads from each ginseng sample were then used to build *de novo* assembly using Trinity (version: trinityrnaseq_r20131110) and TGICL software, producing 63,875 unigenes with pair-end annotation. These assembled unigenes ranged from 300 to 5,931 bp in length, with an average size of 864.86 bp. For

further quantitative assessment of the assembly and annotation completeness, the software tool BUSCO was applied (Fig. 1). Out of 1440 single copy orthologs for plants, our assembly is 72.2% complete (1009 complete single-copy BUSCOs and 30 complete duplicated BUSCOs), while 12.4% of contigs are fragmented (179 BUSCOs) and 15.4% are missing (222 BUSCOs).

The expression level of each unigene was summarized by Bowtie2 (Langmead & Salzberg, 2012) and express (Roberts & Pachter, 2013) software, using the FPKM method for statistical analysis. After removal of lowly expressed genes, Deseqsoftware (Love, Anders, Kim, & Huber, 2015) was used to identify differentially expressed genes (DEGs). With fold change >2 or <0.5 and FDR < 0.05 as screening standards, we found 3,216 DEGs of which 1,190 unigenes were up-regulated and 2,016 were down-regulated in Shizhu ginseng compared to those of Yuan ginseng. The mRNA transcriptome database, which combines available NGS and EST sequences in NCBI databases, makes up the ginseng RNA-seq reference sequences. It was herein used to predict the known and novel miRNAs in *P. ginseng*.

To verify DEGs identified by RNA-Seq, qRT-PCR was performed using the same batch of RNA samples. Among the 17 randomly selected DEGs, which showed the same expression patterns, as evidenced by qRT-PCR (Fig. 2) and RNA-Seq data. Three DEGs in Yuan ginseng were significantly up-regulated, whereas two were significantly down-regulated.

### 3.2. Analysis results of small RNA sequencing

In order to identify small RNAs in different growth year of *P. ginseng*, RNAs isolated from the root tissues of Yuan ginseng and Shizhu ginseng were analyzed by high-throughput Illumina sequencing. Of the 31,131,079 raw reads sequenced from the two miRNA libraries from *P. ginseng* roots, 19, 795, 558 and 24, 154, 579 clean reads were obtained respectively after removing the adaptor, low-quality sequences and those shorter than 18nt or longer than 41 nt. The number of unique clean reads from each of the two libraries exceeded 4,332,290, most of which were sequenced once or twice (about 70% and 15%, respectively), and just 2.6% were sequenced over 10 times, indicating that the small RNAs in *P. ginseng* were large, diverse and highly complicated.

The miRNA size distribution of clean reads in Yuan ginseng and Shizhu ginseng exhibited the highest peaks at 21 nt and 24 nt respectively, in contrast to the secondary peaks at 24 nt and 21 nt. Given that 21 nt and 24 nt are the lengths of most plant miRNAs

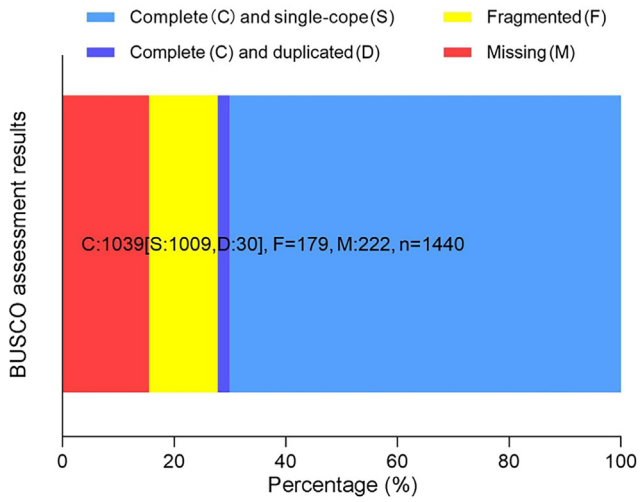


**Fig. 1.** Results of BUSCO assessment.

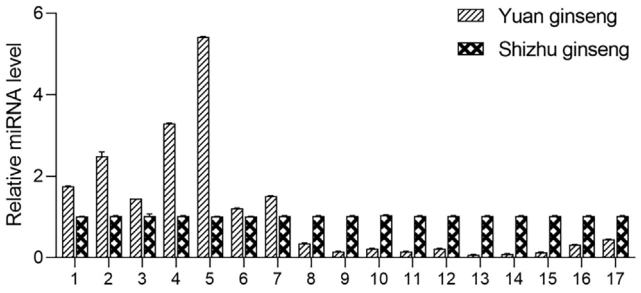


**Fig. 2.** Qrt-pcrvalidation of 17 differentially expressed genes. 1, Trehalose-6-phosphate synthase; 2, Transcription factor TCP15-like; 3, Probable ubiquitin-conjugating enzyme E2 24; 4, Td1TL1 protein, partial; 5, TSPO (outer membrane tryptophan-rich sensory protein)-related; 6, Cell differentiation protein RCD1 like; 7, Growth-regulating factor 4-like; 8, Phosphoenolpyruvate/phosphate translocator; 9, Auxin-induced protein 22D-like; 10, UGT12; 11, Pleiotropic drug resistance transporter; 12, Pyridoxal phosphate (PLP)-dependent transferases superfamily protein; 13, CYP3; 14, Class 1 chitinase; 15, Pathogenesis-related leaf protein 6-like; 16, Endoribonuclease Dicer homolog 1; 17, SPX domain-containing membraneprotein.

and small interfering RNAs respectively, the length distribution of *P. ginseng* miRNAs is highly similar to those of other plant miRNAs (Klevebring et al., 2009). The size distribution analysis of unique small RNA sequences showed that the group of 21nt small RNAs had the highest percentage of miRNAs beginning with a U nucleotide, whereas the 23 nt and 24 nt groups were enriched with sequences having an A at the 5′ end (Fig. 3), which may be attributed to Dicerdigestion.

### 3.3. Identification of known miRNA families

Through mapping the unique miRNA mature sequences to miRBase 19.0 without mismatches, 2,509,667 and 2,679,530 known miRNA sequences encompassing 410 and 406 unique known miRNAs were identified in Yuan ginseng and Shizhu ginseng, respectively. Subsequently, we grouped these known miRNAs into 71 families in the two libraries by mapping small RNAs to the miRNA regions of known precursors, including miR159, miR166, miR168, miR172, miR319, miR396, miR403, miR4376, miR482 and miR2118.

By referencing the whole plant database, 34 of the 71 identified miRNA families were categorized into conserved families. Among them, miR166, miR168, miR159, miR319, miR396 and miR403, which were highly expressed in more than four monocots and dicots, were highly conserved miRNA families. In contrast, miR482, miR158, miR165 and miR391 that were found in two or three monocots and dicots were grouped into less conserved miRNAs. In addition, miR166, miR319, miR396 and miR159 from these conserved miRNA families had 24, 20, 16 and 13 members respectively. The 37 non-conserved miRNA families were also detected from both *P. ginseng* cultivors, the majority of which consisted of a very small number of family members, i.e. seven, three and two for miR6135, miR6136 and miR2118 respectively, and only one for another non-conserved miRNA (Fig. 3).

It has previously been reported that high-throughput sequencing yielded miRNA expression (Szwacka et al., 2018). Of these 34 conserved miRNA families, miR166 was sequenced more than 2,000,000 times, which accounted for 70.2% of total conserved miRNA reads, as the most abundant miRNA family in *P. ginseng* tissues. MiR396, which was sequenced more than 80,967 times, accounted for about 10.0% of total conserved miRNA reads, as the second most abundant miRNA family, followed by miR159 with 78,424 times of sequencing and <8.1% of total conserved miRNA reads. Several miRNA families, including miR168, miR319,

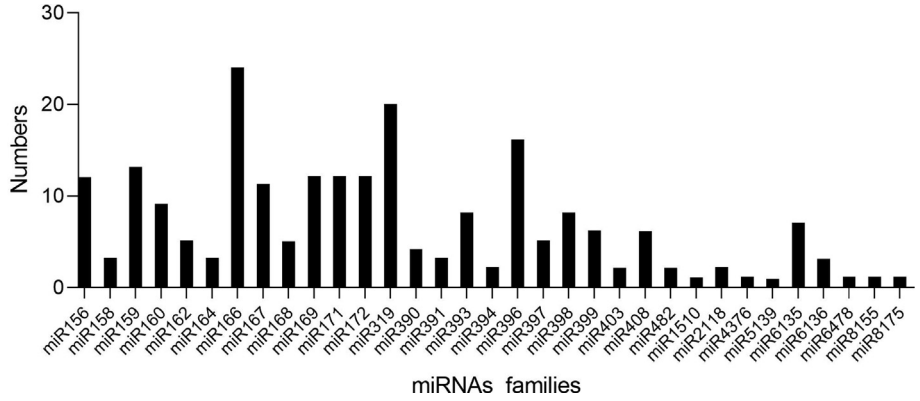


**Fig. 3.** Distribution of known miRNA family members in *P. ginseng*.

miR403, miR482, miR6135 and miR2118, had moderate abundances (Fig. 4A and B). Although miR390, miR394 and miR160 families were highly conserved, the abundances were low (Fig. 4C). Contrarily, a few non-conserved miRNA families, such as miR1140, miR4376, miR5139, miR6136 and miR8155, had lower expression levels of <1,000 reads in both libraries.

Moreover, members in the same miRNA family may have significantly different expression levels, as represented by miR166 members with abundances ranging from 16 to 1,977,056 reads. In short, the expression levels of conserved and non-conserved miRNAs in *P. ginseng* tissues had significant differences, being in agreement with previous studies reporting that non-conserved miRNAs were less expressed than conserved ones (Wu et al., 2012; Mathiyalagan et al., 2013).

With Yuan ginseng as the experimental group and Shizhu ginseng as the control group, we used transcript permillion (TPM) to estimate the expressions of miRNAs (Table 1) in order to screen differential expressions ($P \leq 0.05$) with fold-change >2 or <0.5. As a result, 24 differentially expressed miRNAs were obtained, of which 13 differential miRNAs (diff miRNAs) were up-regulated but the other 11 were down-regulated (Fig. 5). To verify diff miRNAs identified by small RNA sequencing, qRT-PCR was performed

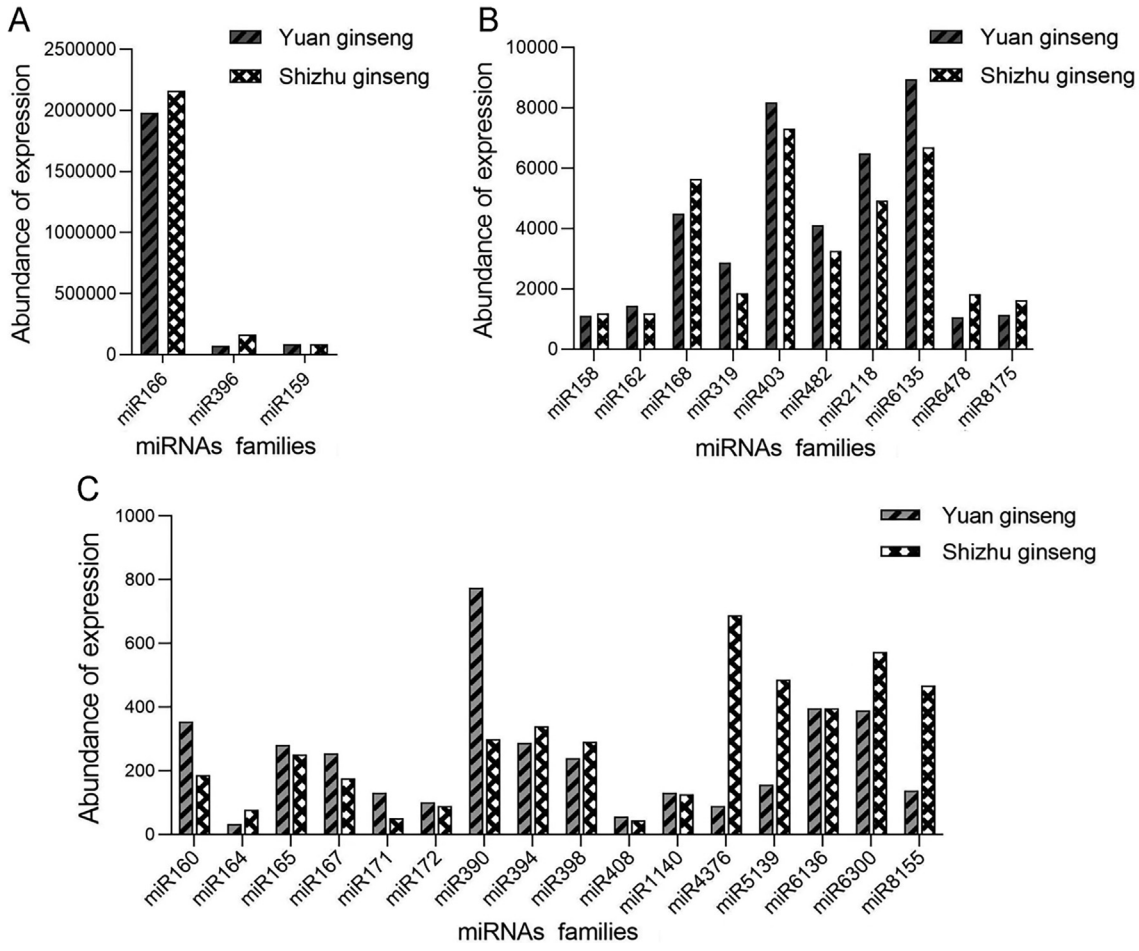


**Fig. 4.** High (A), moderate (B) and (C) low expression abundances of known miRNA families in *P. ginseng*.

**Table 1**
Conserved and non-conserved miRNAs in *P. ginseng*.

| miRNA | | Transcript permillion (TPM) | | miRNA sequence (5′−3′) | Size (nt) | Fold chage |
|---|---|---|---|---|---|---|
| | | Yuan | Shizhu | | | |
| Conserved miRNAs | miR166 | 1,977,056 | 2,170,964 | TCTCGGACCAGGCTTCATTCC | 21 | 1.111 |
| | miR396 | 52,967 | 148,308 | TCTCGGACCAGGCTTCATTCC | 21 | 0.435 |
| | miR159 | 78,424 | 54,781 | TTTGGATTGAAGGGAGCTCTA | 21 | 1.747 |
| | miR403 | 8,220 | 7,324 | TTAGATTCACGCACAAACTCG | 21 | 1.370 |
| | miR168 | 4,482 | 5,646 | TCGCTTGGTGCAGGTCGGGAC | 21 | 0.967 |
| | miR319 | 8,579 | 3,649 | TTGGACTGAAGGGAGCTCCCT | 21 | 2.869 |
| | miR162 | 1,359 | 1,147 | TCGATAAACCTCTGCATCCAG | 21 | 1.446 |
| | miR158 | 1,087 | 1,105 | TTTCCAAATGTAGACAAAGCA | 21 | 1.200 |
| | miR394 | 283 | 333 | TTGGCATTCTGTCCACCTCCAT | 22 | 1.037 |
| | miR390 | 772 | 291 | AAGCTCAGGAGGGATAGCGCC | 21 | 3.237 |
| | miR398 | 230 | 287 | TGTGTTCTCAGGTCGCCCCTG | 21 | 0.977 |
| | miR167 | 277 | 253 | TGAAGCTGCCAGCATGATCTGG | 22 | 1.336 |
| | miR6135 | 8,950 | 6,691 | AATTGGCCAATAGAATACTGACAC1.632 | 24 | 1.632 |
| Non-conserved miRNAs | miR2118 | 6,541 | 4,882 | TTTCCTATTCCACCCATCCCAT | 22 | 1.635 |
| | miR482 | 4,049 | 3,251 | TCTTGCCAATTCCTCCCATTCC | 22 | 1.519 |
| | miR6478 | 971 | 1,813 | CCGACCTTAGCTCAGTTGGTG | 21 | 0.653 |
| | miR8175 | 1,075 | 1,537 | GATCCCCGGCAACGGCGCCA | 20 | 0.854 |
| | miR4376 | 84 | 683 | TGCAGGAGAGATGACGCCCATC | 22 | 0.150 |
| | miR6300 | 386 | 565 | GTCGTTGTAGTATAGTGG | 18 | 0.833 |
| | miR5139 | 142 | 483 | AAACCTGGCTCTGATACCA | 19 | 0.258 |
| | miR8155 | 138 | 463 | TAACCTGGCTCTGATACCA | 19 | 0.364 |
| | miR6136 | 393 | 392 | ACGGGTGAGTAAGATAAGGGGTAT1.223 | 24 | 1.223 |

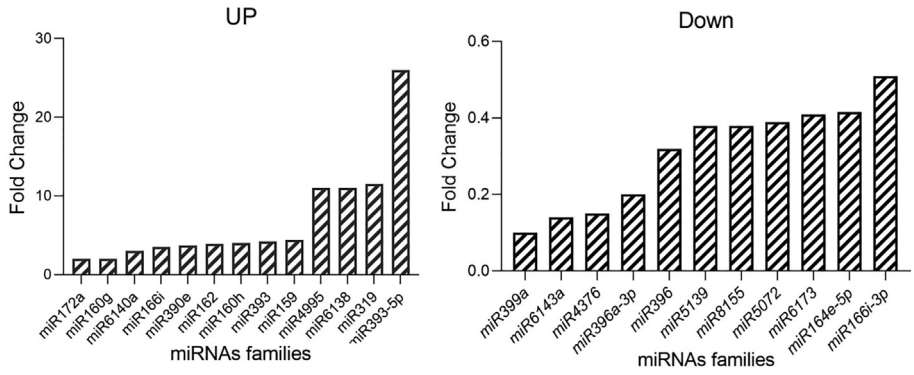


**Fig. 5.** Relative expression levels of known differential miRNAs.

by using the same batch of RNA samples. Nine randomly selected diff miRNAs was evidenced by qRT-PCR (Fig. 6), which showed the same expression patterns with small RNA sequencing data.

### 3.4. Analysis results of degradome sequencing and data summary

Plant miRNAs showed perfect or near-perfect complementarities to their targets (Zhou et al., 2010; Singh et al., 2018). Although a few P. ginseng miRNA targets have been previously predicted (Wu, Wang, Ma, Yuan, & Lu, 2012; Mathiyalagan et al., 2013), none of them have yet been experimentally characterized. In this study, the target genes were predicted, confirmed and analyzed by high-throughput degradome sequencing.

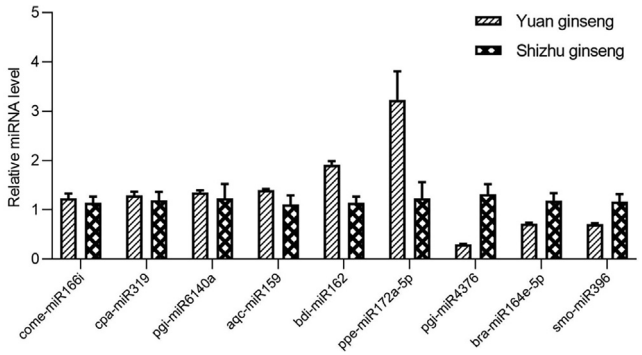


**Fig. 6.** Qrt-pcrvalidation of differential miRNA.

In total, 751,799,075 and 823,042,333 raw reads obtained from Yuan ginseng and Shizhu ginseng respectively generated 15,990,607 and 17,505,975 clean reads from the degradome library. Using Rfam, Genbank and PolyN databases, we retrieved the clean tag sequence information, and remove drRNA, scRNA, snoRNA, snRNA and tRNA that were annotated by Rfam and Genbank. As for the tags not annotated by any database, 1,601,920 and 1,265,753 cDNA-sense tags were ultimately obtained from Yuan ginseng and Shizhu ginseng respectively through comparison with reference genome (unigenes) for the confirmation and analysis of subsequent degradation sites. With Cleaveland 3 software, the computer system visually displayed the apparent peak value of a point in the mRNA sequence, as a candidate miRNA cleavage site of a sliced target. The sliced target transcripts were grouped into five categories as follows based on the relative abundances of degradome signatures at the target mRNA sites. Category 0 is the solitary cleavage site where the most abundant raw tags ($\geq$2) match the transcript. Category 1 is juxtaposed with some other sites in the same transcript, and has two or more raw tags at the position. Category 2 is the position with raw tags ($\geq$2) less than

the maximum but more than the median reads of the transcript. Category 3 has tag abundance (≥2) equal to or less than the median reads of the transcript, and Category 4 means there is only one raw tag at the position.

### 3.5. Identification and annotation of targets for P. ginseng miRNAs

Through degradome sequencing, 13 sliced targets for six known and two novel miRNAs were identified. Except for novel miRNA comp65451 that was grouped into category 2, the other 12, including the targets of aqc-miR159, bdi-miR162, cpa-mi319, cme-miR166i, pgi-miR4376, smo-miR396 and comp46638, all fell into category 0. The identified targets for known and novel *P. ginseng* miRNAs carried annotations of transcription factors, response factor or signal transduction pathway, such as the transcription factor (GAM) family, growth-regulating factor (GRF), domain-containing membrane protein, homeobox-leucine zipper protein, DNA binding protein and TIR-NBS-LRR resistance protein. Nag and Jack (2010) reported that miR319, miR159 and miR396 played important roles in the flower and leaf development of plants. Likewise, we found that miR159 and miR319 of both *P. ginseng* species targeted the MYB domain protein family (GAMYB), and miR396 mainly targeted GRF, being consistent with the results of *Arabidopsis* research. Specifically, auto-inhibited calcium ATPase was identified as the target of pgi-miR4376, a specific and non-conserved miRNA regulating the flowering organs and period of plants. TIR-NBS-LRR resistance protein was targeted by miRNA comp65451, a *P. ginseng* miRNA that was specific but in need of further exploration (Table 2). In addition, a small proportion of these non-conserved and novel miRNAs targeting genes without functional annotations may indicate their unknown special roles in the biological or developmental processes of ginseng. However, miR172, miR168, miR403 and other miRNAs with high expression levels from the degradome sequencing data have been failed to validate the target genes.

## 4. Discussion

### 4.1. MiRNAs in P. Ginseng may have a regulatory effect

In this study, 13 targets of eight miRNA families were determined by bioinformatics method and degradome sequencing. An identical target gene regulated by miR159 and miR319 with the functional annotation of transcription factor GAMYB was verified, and identified as a homologue to *Arabidopsis* target gene MYB101. This also supports the theory that miR159 can inhibit the growth of plants and promote programmed cell death by regulating the signal transduction pathways of ABA and GA (Alonso-Peral et al., 2010). It is well-documented that HD-Zip can increase the abiotic stress in plant growth and development, such as drought and salt stress, as well as resistance to fungal diseases. In our study, cme-miR166 was identified as a miRNA targeting gene encoding cognate leucine zipper protein and DNA binding transcription factor. This may indicate that miR159 and miR166 play a role in regulating the development of whole P. ginseng plant.

As a non-conserved miRNA of P. ginseng, pgi-miR4376 has a target gene with the function annotation of Ca2+-ATP enzyme inhibitor (auto-inhibited calcium ATPase), ahomologue to the Arabidopsis ACA8 target gene which, according to a previous study, was associated with negative regulation of the specific surface area of small cytoplasm in plants by inhibiting its own cell membrane (Wang et al., 2011). The importance of miR162 to the development of whole *P. ginseng* plant was indicated by its significantly up-regulated level as well as its target gene DCL1_ARATH, a homologue to endonuclease Dicer that can induce gene silencing and antiviral action.

Comp65451 was confirmed as a novel miRNA encoding TIR-NBS-LRR with a target of TMVRN_NICGU, which was homologous to miR1885 target gene and closely involved in biological stress responses by altering the auxin expression and distribution in plants (He, Fang, Feng, & Guo, 2008). To sum up, a number of miRNA target genes were successfully identified efficiently by degradome sequencing, with their functions annotated systematically and comprehensively.

Increasing evidence suggests that miRNA can also participate in the biological interaction process through cross-boundary regulation (Zhu et al., 2017). Rice osa-miR162a can enter the brown planthopper through feeding, silence the expression of NlTOR, a key gene for the growth and development of brown planthopper, and affect the expression of the downstream vitelline gene NlVg, thereby negatively regulating the egg production and hatchability of brown planthopper (Shen et al., 2021). Vm-milR1, which is highly expressed during host infection, can positively regulate host disease resistance to apple tree decay bacteria by inhibiting the expression of the host-like receptor protein kinase genes MdRLKT1 and MdRLKT2 (Xu et al., 2022). Therefore, we speculated that miRNA in *P. ginseng* may also play a certain regulatory role. There is an obvious differential expression of miRNA between Linxia ginseng and Yuan ginseng, which may be responsible for the clinical efficacy difference (Peng et al., 2021; Wang et al., 2019).

### 4.2. MiRNA may be a new perspective for differences in efficacy between Shizhu ginseng and Yuan ginseng

Both clinical efficacy and laboratory pharmacology of more than 10 years old Shizhu ginseng are significantly better than 3–5 years

**Table 2**
Identification of ginseng miRNA target gene information by degradome sequencing.

| miRNA | Target gene annotation | Targets | Cleavage site | Score | Category | cDNA length |
|---|---|---|---|---|---|---|
| aqc-miR159 | Transcription factor GAMYB | GAM1_ORYSI | 1,699 | 2.5 | 0 | 2,712 |
| bdi-miR162 | Endoribonuclease Dicerhomolog1 | DCL1_ARATH | 4,288 | 1.5 | 0 | 6,288 |
| cpa-miR319 | Transcription factor GAMYB | GAM1_ORYSI | 1,699 | 1 | 0 | 2,712 |
| cme-miR166 | Homeobox-leucine zipper protein | REV_ARATH | 1,105 | 2 | 0 | 3,470 |
| | DNA binding protein | ATB15_ARATH | 1,635 | 2 | 0 | 3,786 |
| pgi-miR4376 | Auto-inhibited calcium ATPase | ACA8_ARATH | 328 | 3 | 0 | 739 |
| smo-miR396 | Hypothetical protein | GRF1_ARATH | 120 | 2 | 0 | 1,386 |
| | growth-regulating factor | GRF3_ORYSJ | 836 | 2 | 0 | 2,009 |
| | unnamed protein product | GRF4_ORYSJ | 848 | 2 | 0 | 2,239 |
| | growth-regulating factor | GRF6_ORYSJ | 1,107 | 2 | 0 | 2,498 |
| | uncharacterized protein | RPD1_ARATH | 1,849 | 2 | 0 | 3,153 |
| comp46638 | SPX domain-containing membrane protein | SPXM3_ARATH | 376 | 1.5 | 0 | 3,251 |
| comp65451 | Tir-nbs-lrr resistance protein | TMVRN_NICGU | 852 | 1.5 | 2 | 1,834 |

old Yuan ginseng, but the study found that there is no significant difference between the two in the main effective components of ginsenosides and ginseng polysaccharides, indicating that the reasons for the great difference in therapeutic effect of ginseng in different growth years cannot be explained from the aspects of chemical components and compounds.

This study showed that the miRNAs of Shizhu ginseng and Yuan ginseng of different growth years are significantly different. The prediction of the target gene function of their different miRNAs mainly relates to the energy metabolism, immune regulation, tumor and so on, showing that they are consistent with the clinical efficacy range of Shizhu ginseng and Yuan ginseng. It suggests that miRNAs may be one of the active substance components of Shizhu ginseng and Yuan ginseng, providing a new perspective on ginseng research.

## 5. Conclusion

The present study is a report to examine the miRNA expression profiles of *P. ginseng* roots. By using the Illumina HiSeq™2500 sequencing platform, we successfully constructed the RNA-Seq, small RNA-Seq and degradome library, and identified the miRNA species of ginseng, which showed that root of fresh ginseng was rich in miRNA; miRNAs play crucial roles in a wide range of biological processes, including developmental timing, apoptosis, metabolism, hormone signaling, and many others. This study started with the biological functions of miRNAs contained in ginseng itself, and found that there were differential expression miRNAs in different growth years of ginseng, and significant difference of miRNA mainly regulated the expression of DCL, E2-UBC, ACA8, AP2 and other target genes. Further studies are required to investigate the roles of the candidate genes such as the transcription factor, F-Box auxin response factor, TIR-NBS-LRR and so on, which mainly related to energy metabolism and so on in *P. ginseng*. This study suggests that miRNA in ginseng may be one of the effective constituents for the clinical efficacy of ginseng, providing a reference for further research of *P. ginseng*.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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