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Full Paper

Identification of miRNAs responsive to drought stress in herbaceous peony by high-throughput sequencing

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Abstract: To clarify drought-stress-induced molecular changes in herbaceous peony (*Paeonia lactiflora*), we construct small RNA libraries by high-throughput sequencing. A total of 58 known miRNAs and 83 novel miRNAs are identified. Among them, 61 miRNAs are differentially expressed. In addition, differentially expressed miRNAs predict 122 target genes, 68 of which are annotated. When combined with gene ontology enrichment and Kyoto Encyclopedia of Genes and Genomes pathway analysis, 35 miRNAs and 67 target transcripts that might be associated with drought stress are detected. Expression analysis of some miRNAs are performed, which is consistent with the results of targets of transcription factors and functional genes. These results provide a better understanding of the roles of miRNAs under drought stress in herbaceous peony.

Keywords: herbaceous peony, *Paeonia lactiflora*, miRNA, drought stress, quantitative real-time polymerase chain reaction (qRT-PCR)

INTRODUCTION

Drought is a serious problem hindering agriculture and horticulture globally. Drought stress seriously and negatively affects the growth and development of plants, which have evolved complex regulatory mechanisms to adapt to or resist drought stress during long-term natural

selection [1]. With the development of molecular biology, research on plants growing under abiotic stress conditions has reached the level of gene expression, which is mainly regulated at the transcriptional and post-transcriptional levels. At the post-transcriptional level, microRNA (miRNA) is a non-coding RNA of 19-24 nucleotides (nt) in length that regulates gene expression by degrading or inhibiting translation of the target gene (mRNA), and is an important mechanism of a plant's response to abiotic stress [2, 3]. At present, a large number of miRNAs related to drought stress have been identified in many plants by means of high-throughput sequencing, a technique that can compare obtained sequences with known sequences in databases, mine new genes and predict their functions. For example, by using high-throughput sequencing, Liu et al. [4] compared the miRNA profiles before and after drought treatment in two tomato (*Solanum lycopersicum*) genotypes, and a total of 108 conserved and 208 novel miRNAs were identified, among which 32 and 68 showed a significant change in expression after stress. In Chinese white poplar (*Populus tomentosa*), significant changes in the expression of 17 conserved miRNA families and nine novel miRNAs were observed in response to drought stress [5].

Herbaceous peony (*Paeonia lactiflora* Pall.), which belongs to the Paeoniaceae family, is a sought-after cut flower in international markets. Until now, the miRNAs of some herbaceous peony cultivars have been sequenced in the fields of red/yellow bicoloured flowers [6], thermo-tolerance [7], and response to a fungus, *Botrytis cinerea* [8]. In the study of Zhao et al. [6], 207 and 204 conserved miRNAs and 38 and 42 novel miRNAs were identified from red outer petals and yellow inner petals respectively, and these were confirmed by subcloning. Hao et al. [7] chose a thermo-tolerant cultivar 'Zifengyu' and a moderately thermo-tolerant cultivar 'Hongyanzhenghui' under natural long summer heat to investigate heat-responsive miRNAs by miRNA sequencing. They found that 71 known miRNAs with 272 potential target genes and three novel miRNAs with four potential target genes were significantly differentially expressed, suggesting that miR172c-3p, miR395a, miR397a, miR408-5p and miR827, which were up-regulated by heat stress and expressed much higher in thermo-tolerant cultivar 'Zifengyu', might be involved in the response to heat stress. In the study of Zhao et al. [8], a resistant cultivar 'Zifengyu' and a susceptible cultivar 'Dafugui', with significantly different levels of resistance to *B. cinerea* under field conditions, were used as the experimental materials. A total of 237 conserved and seven novel miRNA sequences were differentially expressed between the two cultivars, and they found that miR5254, miR165a-3p, miR3897-3p and miR6450a might be involved in the response of *P. lactiflora* to *B. cinerea* infection.

With a perennial crown (metamorphosed underground shoot), herbaceous peony can adapt to a wide range of ecological conditions. Our research team focused on physiological and biochemical responses of herbaceous peony cultivars to drought stress by analysing changes in 13 stress-related indices including drought damage index, leaf relative water content, substances involved in osmotic adjustment, antioxidant enzymes, and hormones [9]. We also found that the molecular mechanism underlying the response of *P. lactiflora* to drought stress is a very complicated process. Transcriptomics analysis allowed transcription factors such as ethylene responsive element binding

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factor, WRKY and NAC transcription factor family members to be identified from 4,198 differentially expressed genes. These transcription factors are likely involved in the regulation of the biosynthesis of hormones such as indole-3-acetic acid, zeatin, gibberellin and abscisic acid [10]. In this study we utilise high-throughput sequencing technologies to analyse the profiles of differential expression of miRNAs in the leaves of herbaceous peony plants grown in pots with different soil moisture gradients. Conserved and novel miRNAs are identified, target genes for miRNAs are predicted, and the expression patterns of selected miRNAs are examined by quantitative real-time polymerase chain reaction (qRT-PCR).

MATERIALS AND METHODS

According to our previous research [9], *P. lactiflora* 'Karl Rosenfield' has considerable drought resistance and it was used in this study. One hundred three-year-old potted plants were collected from a nursery in Xiaotangshan, Beijing. At the end of September 2016, the dormant plants were pruned to the ground, and the roots were lifted out of the ground and washed to ensure that the growth state of all plant materials were basically the same. Then they were planted with the same mass of substrate (peat: perlite: vermiculite = 3:1:1, v/v) in pots of the same size. In mid-April of 2017 all plants were moved into the greenhouse under the same conditions: light intensity of 30-1000 $mmol \cdot m^{-2} \cdot s^{-1}$; 9 $h \cdot d^{-1}$ photoperiod; 50-75% relative humidity; and day/night temperatures of 26-30 ºC/14-18ºC.

Three drought stress treatments were used to represent a gradient: control (CK), moderate drought stress (MD) and severe drought stress (SD), the soil water content of each treatment being 60 ± 2%, 30 ± 2% and 10 ± 2% respectively of the saturated soil moisture gradient. The division of SMC, which was measured with a FieldScout (TDR 100, Spectrum Technologies, USA), was based on the methods of Guo [11] and Wang et al. [12]. Saturated soil moisture was determined in pre-experiments which began on 10 May 2017. All plants were irrigated thoroughly and watering was then stopped to allow soil to dry naturally. Soil water content was measured with a FieldScout between 8:00-9:00 a.m. every morning. Twelve pots were selected randomly from all plants that reached the corresponding gradient on the same day. Phenotypic traits of herbaceous peony under drought stress have been explained in detail in our previous study [9]. The leaves were collected randomly and mixed, wrapped separately in aluminum foil, placed immediately into liquid nitrogen and stored at -80ºC before RNA extraction, analysis and sequencing.

RNA samples were extracted with Trizol (Life Technologies, USA). RNA purity was checked using a NanoPhotometer spectrophotometer (Implen, USA). With the Qubit RNA assay kit, RNA concentration was measured in the Qubit 2.0 Fluorometer (Life Technologies, USA). RNA integrity was assessed using RNA Nano 6000 assay kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, USA). Sequencing libraries were generated using the NEBNext Ultra small RNA Sample Library Prep kit for Illumina (NEB, USA) following the manufacturer's recommendations. The library preparations were sequenced on an Illumina Hiseq 2500 platform and paired-end reads were generated. Three biological replicates were prepared for each sample.

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After removing raw data, the remaining reads were used to detect known miRNAs and novel miRNAs predicted by comparing with known miRNAs on the miRBase. Expression levels of miRNAs were calculated according to the transcripts/million value using the method of Fahlgren et al. [13]. Differential expression analysis was performed using IDEG6 to obtain differentially expressed miRNAs (DEmiRNAs) between sample pairs [14]. In the screening process a false discovery rate ≤ 0.01, a difference multiple fold change ≥ 2, and a p value ≤ 0.01 were used as screening criteria. Potential targets for the miRNAs were predicted using TargetFinder software. The predicted target gene sequences were found in the transcriptomes of *P. lactiflora* (BioProject accession number: PRJNA573496). These sequences were aligned with non-redundant protein sequence (NR), Swiss-Prot, gene ontology (GO), cluster of orthologous groups of proteins (COG), Kyoto encyclopedia of genes and genomes (KEGG), cluster of orthologous groups for eukaryotic complete genomes KOG, and protein family (Pfam) databases using the basic local alignment search tool (BLAST) software to obtain annotated information [10]. The above steps, as well as the creation of the volcano plot map, heat map and GO annotation classification map, were performed by Biomarker Technologies (China).

In order to verify the reliability of the sequenced data, 10 miRNAs were randomly selected for qRT-PCR validation. The sequences of target genes were also found in BioProject PRJNA573496 of the National Center for Biotechnology Information (NCBI) and used to design the gene-specific primers. At most, one target gene per miRNA was selected. *P. lactiflora Actin* (JN105299) was used as the internal reference gene [15] for this experiment. The miRcute Plus miRNA First-Strand cDNA Synthesis kit (Tiangen, China) was used to generate single-stranded miRNA cDNA by reverse transcription. The expression levels of miRNAs were analysed using the miRcute Plus miRNA qPCR kit (SYBR Green) (Tiangen, China) while the expression of target genes was assessed with the TransStart Top Green qPCR SuperMix (Transgen, China). A Bio-Rad CFX96^{TM} Real-Time System (Bio-Rad, USA) was used for qRT-PCR, and relative gene expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ comparative threshold cycle (Ct) method [16]. Each reaction was repeated three times. The qRT-PCR expression profiles of miRNAs and their target genes were assessed in Excel. All primers used are listed in Table 1.

RESULTS AND DISCUSSION

High-throughput Sequencing of Small RNAs

In order to identify small RNAs and their distribution patterns in herbaceous peony 'Karl Rosenfield' following drought stress treatments, three small RNA libraries containing CK, MD and SD samples were generated and high-throughput sequenced. Raw reads of independent samples were between 12.5-14.6 million and more than 33.9 million clean reads were obtained from the three libraries after removing adaptors and low-quality reads (Table 2). These data, whose BioProject accession number is PRJNA573491, have been deposited in NCBI.

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Table 1. qRT-PCR primers used for mature miRNAs and their target genes

Name of miR	miRNA forward primer (5'-3')	Target gene	Target gene forward primer (5'-3')	Target gene reverse primer (5'-3')
P. lactiflora Actin	GCAGTGTTCCCCAGTATT	*P. lactiflora Actin*	TTATGCCCTTCCTCACGCTATC	GAGCTGCTTTTGGAAGTCTCCA
pla-miR36066_c0_2075	ATGCTCTGTAGCGCATGTGG	c36066.graph_c0	CDS NOT FOUND	CDS NOT FOUND
pla-miR70241_c1_18668	TGGGTTGCATTTCACTTTGATG	c70241.graph_c1	AAAGACCTGGGTAGTGGG	AGCGGATAAGGAAGAAGC
pla-miR70960_c1_20329	CACGTTGATCTTTGCGAGCTC	c70960.graph_c1	CDS NOT FOUND	CDS NOT FOUND
pla-miR72194_c1_24494	ATATTCCCAAGGCCGCGC	c72194.graph_c1	CDS NOT FOUND	CDS NOT FOUND
pla-miR77050_c0_29064	TTCTGGGCAAGACGCATTG	c77050.graph_c0	CAATTTGTTTGTTGGTGCTG	ACTTATCTGATGCGAGGC
aau-miR396	GGTTCCACAGCTTTCTTGAACTG	c69626.graph_c4	GGTTTTACAGACTCATTG	TTACAAATCATAAAAGGG
aly-miR164a-5p	TGGAGAAGCAGGGCACGTGCA	c32727.graph_c0	GTTCCCATAACCACCGATAC	CCTTCAGAACCCTGGTAAAAC
bdi-miR166e-3p	GGACCAGGCTTCATTCCCA	c67201.graph_c1	TTTAATGGAGGAGAATGATAGG	TCACCACCGACTCACAGC
bra-miR164e-5p	GAAGCAGGGCACGTGCAA	c29809.graph_c0	GAGCCTTACTTGAAACCCAT	GATTTCCGTGTTCACATCAGT
gma-miR390b-5p	AAGCTCAGGAGGGATAGCACC	c49972.graph_c0	CDS NOT FOUND	CDS NOT FOUND

Note: Reverse primers of miRNAs were obtained from the miRcute Plus miRNA qPCR kit (SYBR Green) (FP411, Tiangen, China).

Table 2. Statistics of sample sequencing data

Sample	No. of raw read	Length <18 nt	Length > 30 nt	No. of clean read	Q30 (%)
CK	14,625,610	2,488,562	417,220	11,719,813	97.90
MD	12,501,196	1,662,837	444,803	10,393,536	98.08
SD	14,024,062	1,465,603	709,322	11,849,137	97.78

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Identification of Known miRNAs and Novel miRNAs

A total of 58 known miRNAs and 83 novel miRNAs (prefixed with pla-, referring to *P. lactiflora*) were identified in the three libraries (Table 3). These miRNAs are highly conserved among species, and based on sequence similarity, miRNA family analysis of known miRNAs and novel miRNAs was performed to study the conservation of miRNAs in evolution. The results of the analysis show that 88 miRNAs belong to 38 families, and that the MIR396 family has the most miRNAs (8), followed by MIR166 (7) and MIR172 (6). MiR396 is a conserved gene family that is found in many plant species, and some growth-regulating factor (GRF) family genes are generally recognised as their own target genes [17]. The miR396-GRF module plays regulatory roles in plant growth and development and responds to various environmental stresses [18].

Table 3. Summary of miRNA statistics for each sample

Sample	Known miRNAs	Novel miRNAs	Total miRNAs
CK	52	79	131
MD	56	81	137
SD	54	77	131
Total	58	83	141

Analysis of Differentially Expressed miRNAs under Drought Stress

In a comparison of MD and SD treatments with CK, a total of 61 DEmiRNAs were detected, from which 27 known miRNAs and 34 novel miRNAs were analysed by hierarchical clustering analysis. The miRNAs with the same or similar expression behaviour were clustered. These results are shown in Figure 1. According to the negative regulation relationship between miRNA and a target gene [19], it can be inferred that the expression of many target genes regulated by miRNA in response to drought stress is down-regulated. These genes may be involved in providing resistance to drought stress. Among them, 19 miRNAs are always differentially expressed in CK vs MD and CK vs SD. Four genes that are downgraded are aly-miR164a-5p, bra-miR164e-5p, gma-miR164b and gma-miR390b-5p.

miRNA Target Gene Prediction and Functional Enrichment Analysis

The results of target gene prediction are shown in Table 4. In the bioinformatics prediction analysis the relationships between miRNAs and their targets are not one-to-one since many miRNAs can co-target one gene. On the other hand, one miRNA can also have multiple targets [20]. A total of 93 and 167 target genes are predicted by 51 known miRNAs and 60 novel miRNAs respectively, indicating that the novel miRNAs predict more target genes than known miRNAs. Among the 256 target genes, 140 have annotation information, accounting for 54.69%. A total of 122 target genes are predicted by 61 DEmiRNAs, 68 of which are annotated. Among them, there are 31 target genes in the CK vs MD comparison and 58 target genes in the CK vs SD comparison

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(Table 5), while 21 target genes are repeated in both comparisons.

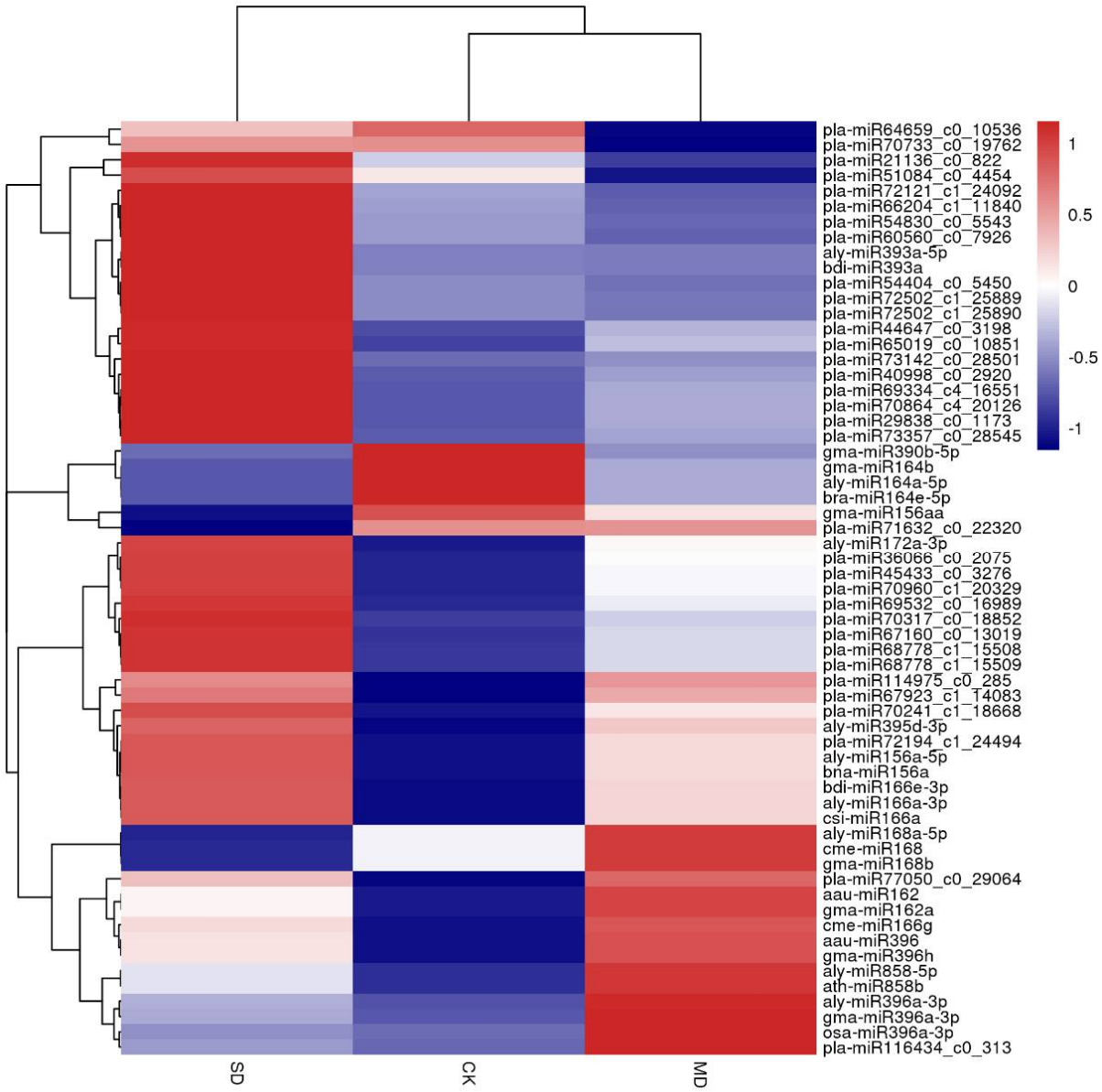

Figure 1. Heat map of hierarchical clustering analysis of DEmiRNAs in libraries of herbaceous peony leaves. Values are normalised by row z-score for clustering. The prefix pla- indicates novel miRNAs from *P. lactiflora*.

Table 4. Number of miRNAs through target prediction

No.	miRNA			Sample		
	Known miRNAs	Novel miRNAs	Total miRNAs	CK	MD	SD
All miRNAs	58	83	141	131	137	131
miRNAs with targets	51	60	111	103	109	106
Target genes	93	167	256	246	253	249

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Table 5. Statistical analysis of DEmiRNA target gene annotation

DEmiRNA comparison	COG	GO	KEGG	KOG	Pfam	Swissprot	eggNOG	NR	Total
CK vs MD	13	17	8	13	26	21	27	31	31
CK vs SD	16	25	17	23	44	44	47	56	58

We classified the functions of miRNA target genes that were differentially expressed using GO analysis with three major categories: biological processes, cellular component and molecular function. The statistical results of GO annotation classification of DEmiRNA target genes between different samples are shown in Figure 2. The target genes of miRNAs, which are differentially expressed both in CK vs MD and in CK vs SD comparisons, are annotated into 28 identical GO terms, among which the higher proportions of annotation are: metabolic process, cellular process and single-organism process (biological processes), cell, cell part and organelle (cellular components), and binding and catalytic activity (molecular functions). Enrichment analysis of DEmiRNA target genes between different samples was performed (enrichment significance <0.05) and the final results are shown in Table 6. Under different drought stresses, only eight GO terms are enriched from the two DEmiRNA target gene sets, and two are not simultaneously enriched in the same item. The intrinsic component of membrane, the integral component of membrane and the membrane which belongs to the cellular component are the most abundantly enriched DEmiRNA target genes.

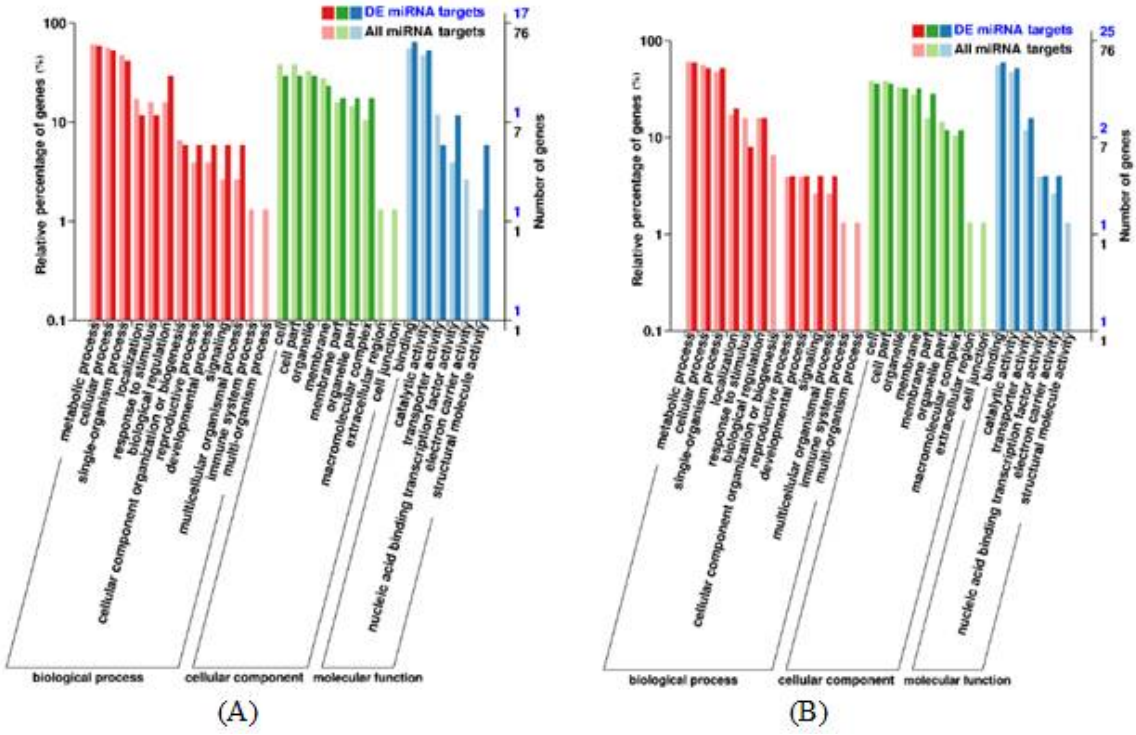

Figure 2. GO annotation classification statistics of DEmiRNA target genes between different samples, i.e. CK vs MD (A), and CK vs SD (B)

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Table 6. GO enrichment of DEmiRNA target genes

GO classification	GO term	No. of DEmiRNA target genes/all genes annotated in this term	
		CK vs MD	CK vs SD
biological processes	biological regulation	5/11	——
biological processes	cellular biosynthetic process	6/19	——
biological processes	regulation of metabolic process	4/8	——
cellular component	integral component of membrane	——	7/11
cellular component	intrinsic component of membrane	——	7/11
cellular component	membrane	——	7/20
molecular function	DNA binding	5/7	——
molecular function	transferase activity	6/13	——

KEGG annotation was performed in the pathways of DEmiRNA target genes between different samples. These results are shown in Table 7. As can be seen in the table, the target genes of the two sets are associated with a total of six KEGG pathways, and both sets are annotated to the secondary-pathway spliceosome and ribosome, which are under the Genetic Information Processing class. By calculating the enrichment factor and enrichment significance ($q \leq 0.1$), one KEGG enrichment pathway is obtained, i.e. photosynthesis of CK vs. SD, which is linked to the determination of subsequent photosynthetic indicators.

Table 7. KEGG pathways of DEmiRNA target genes

DEmiRNA comparison	KEGG pathway	Pathway ID
CK vs MD	Ribosome	ko03010
	Spliceosome	ko03040
CK vs SD	Starch and sucrose metabolism	ko00500
	Spliceosome	ko03040
	Phagosome	ko04145
	Ribosome	ko03010
	Phosphatidylinositol signalling system	ko04070
	Photosynthesis	ko00195

According to DEmiRNAs, miRNA target gene prediction, GO and KEGG annotations and their enrichment analysis, we list miRNAs and their target genes that might be associated with drought stress in herbaceous peony, including 35 miRNAs and 67 target transcripts (Table 8). These targets can be divided into two groups. The first group is that of transcription factors. In addition, c67894.graph_c0, the target gene of pla-miR70241_c1_18668, is annotated as *bHLH87*. The basic helix-loop-helix (bHLH) transcription factor in plants plays a principal role in developmental

processes that might govern biotic and abiotic stress responses [21,22]. Wang et al. [23] found that a grape (*Vitis vinifera*) bHLH transcription factor gene, *VvbHLH1*, increases the accumulation of flavonoids and enhances salt and drought tolerance in transgenic *Arabidopsis thaliana*. Kavas et al. [24] identified 155 bHLH protein-encoding genes (*PvbHLH*) by using *in silico* comparative genomics tools. Totally, 100 *PvbHLH* genes targeted by 86 plant miRNAs were identified, and miR396, miR530 and miR165 were one of the most important miRNAs found by targeting and BLAST analysis.

As the target of gma-miR156aa, c61025.graph_c0 is annotated as *TCP11* in our study. TCP (teosinte branched1/cycloidea/proliferating cell) genes encode plant-specific transcription factors and proteins of the TCP family take part in the regulation of many biological processes during plant growth and development, including plant architecture, leaf morphogenesis, hormone pathways and response to environmental stimuli [25, 26]. Viola et al. [27] found that the expression of a repressor form of *AtTCP11* causes pleiotropic developmental alterations and influences the growth of leaves, stems and petioles, and pollen development. In cassava (*Manihot esculenta*), Lei et al. [28] considered that *MeTCP3a* and *MeTCP4*, targets of miRNA319, have altered expression patterns under cold, drought and salt stress, suggesting that miRNAs might participate in distinct signalling pathways and that these genes might play important roles under abiotic stress conditions.

Another group of target genes related to drought tolerance is that of functional genes such as calmodulin (CaM) and pentatricopeptide repeat (PPR). Many abiotic stimuli like drought and salt stresses elicit changes in intracellular calcium levels that serve to convey information and activate adaptive response. Ca^{2+} signals are perceived by different Ca^{2+} sensors and calmodulin is one of the best-characterised Ca^{2+} sensors in eukaryotes [29]. In our research c42372.graph_c0, the target gene of gma-miR156aa (a gene of MIR159 family), is annotated as CaM. The miR159 was found to be less abundant in the salt-tolerant cultivar of finger millet (*Eleusine coracana* L.) during salinity stress, which was predicted to target key salinity-tolerance-related genes, viz. MYB (v-myb avian myeloblastosis viral oncogene homolog) transcription factor and calmodulin-related calcium sensor protein [30]. A rice calmodulin-like gene, *OsMSR2*, enhances drought and salt tolerance and increases abscisic acid sensitivity in *Arabidopsis* [29], so that we may infer that by regulating CaM, gma-miR156aa might be related to response to drought stress in *P. lactiflora*. Both are likely related to abscisic acid, but the manner in which PPR proteins regulate plant response to abiotic stresses is not the same as CaM [31, 32].

In our research three miRNAs, gma-miR396h, pla-miR44647_c0_3198 and pla-miR45433_c0_3276, target three different genes which are all annotated as PPR proteins. The PPR protein family is one of the largest families of land plants and plays important roles in regulating plant response to abiotic stresses, such as in the response of *Arabidopsis* to drought, salt and cold stresses [31] and in rice under salinity or drought stress [33]. Under different abiotic stress conditions, PPR proteins, as target genes, are annotated by ptc-miR475 and ptc-miR476 in poplar (*Populus trichocarpa*) [34]. In tomato (*Solanum lycopersicum*) they are annotated by sly-miR172a [35] and in wheat (*Triticum aestivum* cv. Chinese Spring), by tae-miR2020b-1 [36].

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Table 8. miRNAs and their target genes that might be associated with drought stress in herbaceous peony

Name of miRNA	Target gene	Annotation
aau-miR396, gma-miR396h	c66246.graph_c0	Growth-regulating factor 4 isoform X2
aau-miR396, gma-miR396h	c78627.graph_c0	Uncharacterised protein TCM_024890
aly-miR164a-5p, gma-miR164b	c32727.graph_c0	Amino acid transport and metabolism
aly-miR164a-5p, bra-miR164e-5p, gma-miR164b	c29809.graph_c0	NAC domain protein, IPR003441
aly-miR166a-3p, bdi-miR166e-3p, cme-miR166g	c67201.graph_c1	Homeobox-leucine zipper protein ATHB-15
aly-miR396a-3p, osa-miR396a-3p	c71313.graph_c1	Probable histone H2B.3
aly-miR858-5p, ath-miR858b	c49872.graph_c1	Myb-like DNA-binding domain
aly-miR858-5p, ath-miR858b	c66075.graph_c3	O-Glycosyl hydrolases family 17 protein
gma-miR162a	c84714.graph_c0	Ankyrin repeat-containing protein At2g01680-like isoform X3
gma-miR164b	c66998.graph_c4	Phosphatidate cytidylyltransferase activity
gma-miR164b	c69991.graph_c0	NAD dependent epimerase/dehydratase family
gma-miR396a-3p	c69132.graph_c0	Posttranslational modification, protein turnover, chaperones
gma-miR396h	c105813.graph_c0	Signal transduction mechanisms; Uncharacterised protein LOC100804062
gma-miR396h	c71999.graph_c1	Pentatricopeptide repeat-containing protein At1g31430
aly-miR156a-5p, bna-miR156a	c70414.graph_c2	Squamosa promoter-binding-like protein 16
aly-miR156a-5p, bna-miR156a	c33055.graph_c0	Promoter-binding protein SPL9
aly-miR156a-5p, bna-miR156a	c67012.graph_c0	Promoter-binding protein SPL10
aly-miR156a-5p,	c61916.graph_c1	Squamosa promoter-binding-like protein 18

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Table 8. (Continued)

Name of miRNA	Target gene	Annotation
bna-miR156a		
aly-miR156a-5p, bna-miR156a	c53396.graph_c0	Putative squamosa promoter-binding protein
aly-miR172a-3p	c62314.graph_c1	Ethylene-responsive transcription factor RAP2-7 isoform X2
aly-miR172a-3p	c70440.graph_c4	Inorganic ion transport and metabolism
bna-miR156a	c68327.graph_c2	Hypothetical protein VITISV_042891
gma-miR156aa	c61025.graph_c0	Transcription factor TCP11
gma-miR156aa	c62014.graph_c0	Secondary metabolites biosynthesis, transport and catabolism
gma-miR156aa	c67469.graph_c1	ABC-2 type transporter
gma-miR156aa	c55795.graph_c0	Thylakoid membrane phosphoprotein 14 kDa, chloroplastic
gma-miR156aa	c42372.graph_c0	Calmodulin
gma-miR156aa	c18642.graph_c1	G-box binding factor
gma-miR156aa	c69421.graph_c0	Auxin-induced 5NG4
gma-miR156aa	c63844.graph_c0	GDP-fucose protein O-fucosyltransferase
gma-miR156aa	c109001.graph_c0	U-box domain-containing protein 32
gma-miR156aa	c63143.graph_c0	Hypothetical protein ARALYDRAFT_910959
gma-miR156aa	c54369.graph_c0	Protein transport protein SEC61 gamma subunit
gma-miR156aa	c66544.graph_c0	Protein ENHANCED DISEASE RESISTANCE 2 GN=EDR2
aly-miR168a-5p, cme-miR168, gma-miR168b	c65487.graph_c0	Protein argonaute 1 GN=AGO1
aly-miR395d-3p	c71358.graph_c0	Sulfate transporter family
pla-miR64659_c0_10536	c64659.graph_c0	Hypothetical protein MIMGU_mgv1a019104mg
pla-miR70241_c1_18668	c121123.graph_c0	Zinc-binding
pla-miR70241_c1_18668	c71531.graph_c2	Hypothetical protein PRUPE_ppa017790mg
pla-miR70241_c1_18668	c43202.graph_c0	ABC transporter D family member 2, chloroplastic
pla-miR70241_c1_18668	c38161.graph_c0	Uncharacterised protein LOC104733506
pla-miR70241_c1_18668	c71236.graph_c1	Katanin p80 WD40 repeat-containing subunit B1 homolog
pla-miR70241_c1_18668	c46286.graph_c0	Putative leucine-rich repeat receptor-like serine/threonine-protein kinase At2g24130
pla-miR70241_c1_18668	c57830.graph_c0	Basic helix-loop-helix DNA-binding superfamily

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Table 8. (Continued)

Name of miRNA	Target gene	Annotation
		protein isoform 2
pla-miR70241_c1_18668	c60092.graph_c0	Coenzyme transport and metabolism; Methyltransferase domain
pla-miR70241_c1_18668	c65091.graph_c0	RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain); Heterogeneous nuclear ribonucleoprotein A1/A3 (A)
pla-miR70241_c1_18668	c64647.graph_c0	Serine/threonine-protein phosphatase 7 long form homolog
pla-miR70241_c1_18668	c67894.graph_c0	Transcription factor bHLH87
pla-miR70241_c1_18668	c71020.graph_c0	Vacuolar protein sorting-associated protein 35B-like
pla-miR70733_c0_19762	c66462.graph_c0	60S ribosomal protein L18a isoform X1
pla-miR70733_c0_19762	c58403.graph_c0	Serine/threonine-protein kinase-like protein ACR4
pla-miR51084_c0_4454	c69047.graph_c0	Uncharacterised protein LOC100245992 isoform X3
pla-miR51084_c0_4454	c64764.graph_c0	DNA (cytosine-5)-methyltransferase DRM2-like
pla-miR29838_c0_1173	c69609.graph_c0	Ribonuclease 2-5A
pla-miR29838_c0_1173	c70661.graph_c0	Spermatogenesis-associated protein 20-like
pla-miR44647_c0_3198	c68961.graph_c0	Pentatricopeptide repeat-containing protein At1g12620
pla-miR67160_c0_13019	c73110.graph_c0	Ribonuclease S-7 (Precursor)
pla-miR70317_c0_18852	c50030.graph_c0	RNA-binding protein Nova-1, putative
pla-miR70864_c4_20126	c70864.graph_c4	Cell wall/membrane/envelope biogenesis; Sucrose-phosphate synthase family protein isoform 2
pla-miR72121_c1_24092	c69169.graph_c0	Endonuclease
pla-miR72121_c1_24092	c72121.graph_c1	Photosynthetic reaction center protein; maturase K (chloroplast)
pla-miR69334_c4_16551	c72149.graph_c0	NADPH-cytochrome P450 reductase 1
pla-miR69334_c4_16551	c58538.graph_c0	Hypothetical protein PRUPE_ppa016496mg
pla-miR66204_c1_11840	c66204.graph_c1	Conserved hypothetical protein
pla-miR45433_c0_3276	c72706.graph_c0	Photosystem I P700 chlorophyll A apoprotein A2 (chloroplast)
pla-miR45433_c0_3276	c71022.graph_c0	Pentatricopeptide repeat-containing protein At1g07740, mitochondrial
pla-miR45433_c0_3276	c47204.graph_c0	30S ribosomal protein S14

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qRT-PCR Validation of miRNAs and Corresponding Target Genes

To validate the reliability of the sequencing results, 10 miRNAs (five known and five novel miRNAs) were randomly selected for expression pattern analysis using qRT-PCR. As shown in Figure 3, compared with MD samples, the expression levels of pla-miR77050_c0_29064, aau-miR396, aly-miR164a-5p, bra-miR164e-5p and gma-miR390b-5p are down-regulated while other five miRNAs are up-regulated in SD samples, which is consistent with the results from high-throughput sequencing (Figure 1). Besides, the expression profiles of six target genes, pla-miR70241_c1_18668, pla-miR77050_c0_29064, aau-miR396, aly-miR164a-5p, bdi-miR166e-3p and bra-miR164e-5p were analysed (Figure 4). Except for bdi-miR166e-3p and its

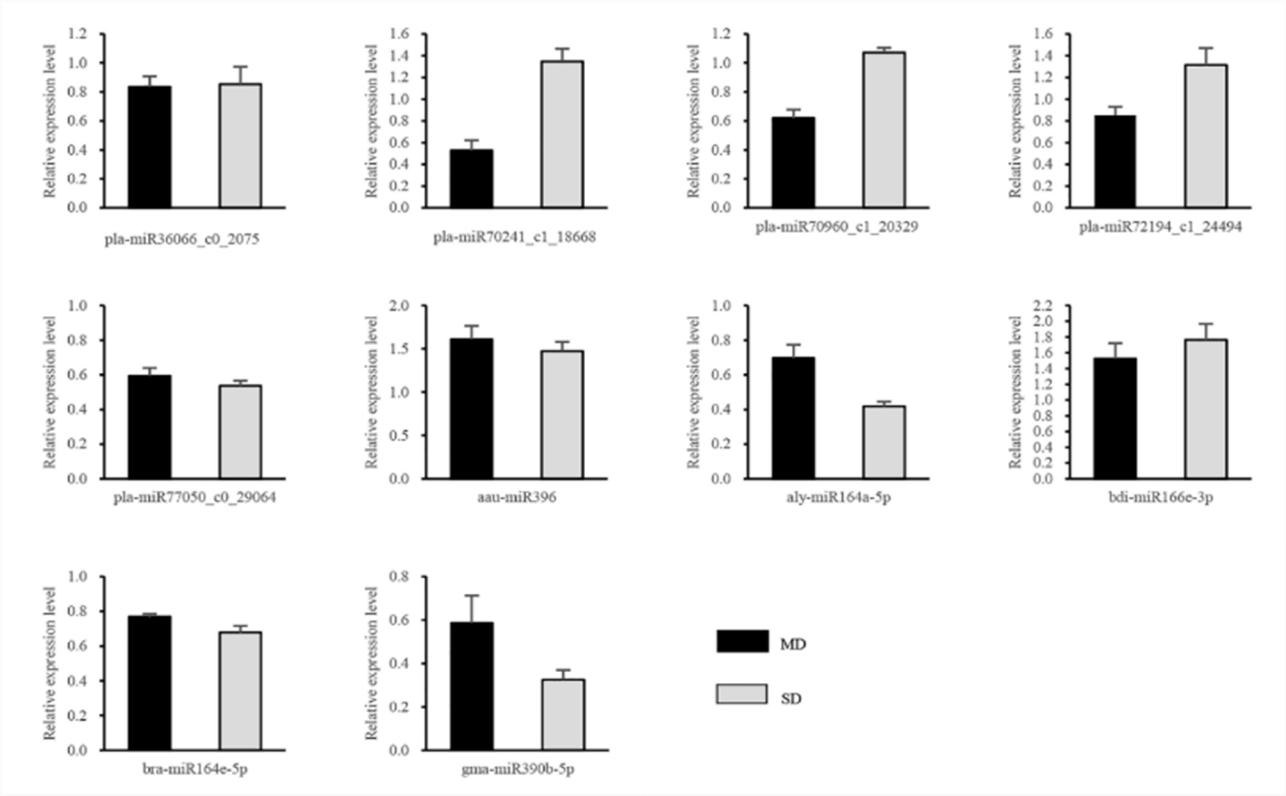

Figure 3. Expression profiles of miRNAs by qRT-PCR

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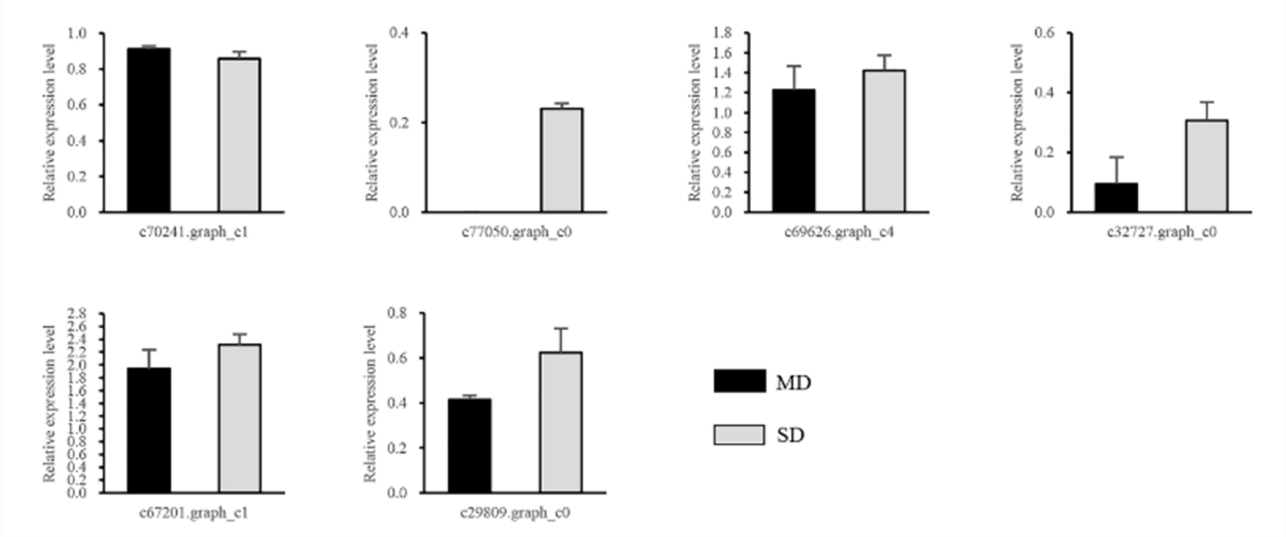

Figure 4. Expression profiles of target genes by qRT-PCR. Note: the target gene CDS of four miRNAs could not be found and/or sequenced in mRNA high-throughput sequencing, so they could not be validated by qRT-PCR (see Table 1).

target gene, their expression levels are opposite to the corresponding target genes according to the qRT-PCR data, suggesting the miRNA-mediated regulation of target gene expression. Among the various comparisons, the difference in relative expression level of pla-miR70241_c1_18668 in the MD vs SD comparison is the largest (0.818), but the difference of its target gene (c70241.graph_c1) is minimum (0.055).

CONCLUSIONS

High-throughput sequencing technology has been performed to characterise the miRNAs responsive to drought stress in herbaceous peony. A total of 58 known miRNAs and 83 novel miRNAs have been identified. Among them, 35 miRNAs and 67 target genes that might be associated with drought stress were listed. This work could offer conjoint analysis materials to reveal the molecular mechanisms of the response of *P. lactiflora* to drought stress and lay a foundation for gene clones related to the drought tolerance of plants.

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