Identification of MicroRNAs and Their Targets Involved in *Paeonia rockii* Petal Variegation Using High-throughput Sequencing

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**ABSTRACT.** Tree peony (*Paeonia* sp.) is a popular traditional ornamental plant in China. Among the nine wild species, *Paeonia rockii* displays wide-ranging, deep purple variegation at the base of the petals, whereas *Paeonia ostii* exhibits purely white petals. Overall, the posttranscriptional regulation involved in tree peony flower opening and pigmentation remains unclear. To identify potential microRNAs (miRNAs) involved in flower variegation, six small RNA libraries of *P. ostii* and *P. rockii* petals at three different opening stages were constructed and sequenced. Using Illumina-based sequencing, 22 conserved miRNAs and 27 novel miRNAs were identified in *P. rockii* and *P. ostii* petals. Seventeen miRNAs were differentially expressed during flower development, and several putative target genes of these miRNAs belonged to transcription factor families, such as Myb domain (MYB), and basic helix-loop-helix (bHLH) transcription factors. Furthermore, an integrative analysis of the expression profiles of miRNAs and their corresponding target genes revealed that variegation formation might be regulated by miR159c, miR168, miR396a, and novel miR_05, which target the MYB transcription factors, chalcone synthase (CHS), and ABC transporters. Our preliminary study is the first report of miRNAs involved in *Paeonia* flower pigmentation. It provides insight regarding the molecular mechanisms underlying the regulation of flower pigmentation in tree peony.

**ADDITIONAL INDEX WORDS.** tree peony, flower coloration, miRNA, MYB transcription factor, chalcone synthase, ABC transporters

Petal variegation is a distinctive trait of flowers from many angiosperm families. Variegation not only attracts pollinators but also improves the ornamental and commercial value of flowers. Tree peony (*Paeonia suffruticosa*), which belongs to the section *Mutan* of the genus *Paeonia* and family Paeoniaceae, is a popular traditional ornamental and medicinal woody shrub native to China; it is known as “the king of flowers” due to its large and colorful flowers (Li, 1999). There are nine wild species of tree peony, including *Paeonia rockii*, *P. ostii*, *Paeonia
delavayi*, *P. potanini*, and *P. ludlowii*. *P. rockii* exhibits wide, deep purple variegation at the base of the petals (Li et al., 2009; Wang et al., 2000). Furthermore, *P. rockii* is an ideal material for studying the mechanism by which variegation occurs in woody plants. Petal variegation has been shown to develop through the differential regulation of enzymes involved in anthocyanin biosynthesis, which has been extensively studied (Espley et al., 2007; Koes et al., 2005). Furthermore, the structural genes involved in anthocyanin biosynthesis are regulated by the
R2R3-MYB, basic helix-loop-helix (bHLH), and WD40 protein transcription factors (TFs), separately or cooperatively (Gonzalez et al., 2008; Koes et al., 2005). Moreover, the R2R3-MYB TFs are considered the main regulators of petal variegation (Albert et al., 2011; Hsu et al., 2015; Martins et al., 2016; Schwinn et al., 2006; Shang et al., 2011; Suzuki et al., 2016; Yamagishi et al., 2010, 2014; Yamagishi, 2016, 2018; Yuan et al., 2014). In tree peony, purple spot pigmentation is the primary effect of the coexpression of chalcone synthase (PsCHS), flavonoid 3′-hydroxylase gene (PsF3′H), dihydroflavonol 4-reductase gene (PsDFR), and anthocyanidin synthase (PsANS) (Zhang et al., 2015). It has been shown to be regulated by R2R3-MYB TFs interacting with a bHLH and a WD40 protein in a regulatory complex that directly activates PsCHS expression (Gu et al., 2018; Shi et al., 2017).

Plant microRNAs, which are small nonprotein-coding single-stranded RNAs with a length ranging from 18 to 25 nt, have key roles in the posttranscriptional regulation of plant growth, cell differentiation, phytohormone signaling, and abiotic and biotic stresses (Axtell et al., 2007; Sunkar et al., 2007). In Arabidopsis thaliana, miRNA156 regulates the squamosal promoter binding protein-like gene (SPL9), which is a negative regulator of anthocyanin accumulation (Gu et al., 2011), and miRNA828 downregulates anthocyanin accumulation through its target genes AtTAS4-siR81(–) and R2R3-MYB TFs (Luo et al., 2012; Nishihara and Nakatsuka, 2011; Tanaka et al., 2008; Yang et al., 2009). Similar results have been reported for Canna indica, tomato (Solanum lycopersicum), and apple (Malus ×domestica) exocarp (Jia et al., 2015; Qu et al., 2016; Roy et al., 2016; Xia et al., 2012). For apple, in addition to three predictable MYBs, MdTAS4-siR81(–) also targets a bHLH TF that interacts with MdMYB10 to regulate anthocyanin biosynthesis (Xia et al., 2012). Moreover, the miR156-SPLs module is conserved in anthocyanin biosynthesis in Litchi chinensis and P. lactiflora (Liu et al., 2017; Zhao et al., 2017). Although miRNAs have been investigated from the flower buds and seeds of tree peony cultivars, no systematic studies exploring miRNAs in the petals of P. ostii and P. rockii have been performed (Yin et al., 2018; Zhang et al., 2018).

In the present study, we analyzed the different miRNAs expressed in the petals of P. rockii and P. ostii at three different opening stages and predicted the putative target genes of the candidate miRNAs. We also characterized the miRNAs and investigated their expression profiles during flower pigmentation and flower opening. Our results could lay the foundation for elucidating the molecular mechanisms underlying tree peony flower pigmentation and may serve as the primary foundation for further studies investigating metabolic regulatory networks.

**Materials and Methods**

**Plant materials.** The P. rockii (PR) and P. ostii (PO) plants were grown under field conditions at Northwest A&F University, Yangling, China. The petal samples were separately detached from plants at five different opening stages (S1, S2, S3, S4, and S5) during the morning in Apr. and May 2016 (Fig. 1). The flower opening stages of PO and PR were as follows: S1, unpigmented tight bud; S2, slightly soft bud without pigmentation; S3, initially open flower with slight pigmentation; S4, half-open flower with slight pigmentation; and S5, fully open and pigmented flower with exposed anthers. To avoid the effects of other organisms (e.g., arthropods, bacteria, or fungi) on the miRNAs and gene expression, only healthy, undiseased, and nonpest-injured flowers were collected. The petals were separately collected from five different flowers from five plants at five different opening stages, washed three times with distilled water, immediately frozen in liquid nitrogen, and stored at −80°C until RNA extraction.

**Small RNA library preparation and sequencing.** Total RNAs from a mixture of petals from five different flowers from five PR and PO individuals at the S1, S3, and S5 stages were isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA), and the resulting samples were denoted as PO-S1, PO-S3, PO-S5, PR-S1, PR-S3, and PR-S5. The quality of the purified RNA was initially assessed by 2% agarose gel electrophoresis and a spectrophotometer (NanoDrop 8000; Thermo Scientific, Waltham, MA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system.

![Fig. 1. Photographs of Paeonia ostii and Paeonia rockii during different flower opening stages. (A–E) P. ostii at S1–S5. (F–J) P. rockii at S1–S5 (S1 = unpigmented tight bud; S2 = slightly soft bud without pigmentation; S3 = initially open flower with slight pigmentation; S4 = half-open flower with slight pigmentation; S5 = fully open and pigmented flower with exposed anthers).](image-url)
Only RNA samples with a 260/280 ratio between 1.8 and 2.0 as well as an RNA integrity number >8.0 were used for cDNA library preparation. Six small RNA (sRNA) libraries were constructed and subjected to single-end sequencing using the Illumina HiSeq 2500 platform (Illumina, San Diego, CA) at Biomarker Technologies (Beijing, China). First, sRNAs (18–30 nt) were isolated from the total RNA by 15% TBE–urea denaturing polyacrylamide gels electrophoresis, and a 5′ RNA adaptor (GTTTACAGGTT CTACAGTCCGACGATC) and 3′ RNA adaptor (TCTGCA CACGAGAAAGCTAGA) were ligated to these sRNAs, which were further reverse-transcribed into cDNAs using the NEB-Next Ultra RNA Library Prep Kit for Illumina (NEB, Ipswich, MA) according to the protocol provided by the manufacturer. These prepared cDNAs were amplified by polymerase chain reaction (PCR) and subjected to sequencing using the Illumina HiSeq 2500 platform. The sequencing analysis was performed using an Illumina Genome Analyzer according to the manufacturer’s instructions. All sequencing data were deposited into the National Center for Biotechnology Information (NCBI, Bethesda, MD) database Sequence Read Archive with accession number SRP102723.

Small RNA analysis and miRNA prediction. After removing reads containing adapters or poly-N and low-quality reads, unique sequences of 18 to 30 nt were used for further analysis. These unique sequences were BLASTn-searched against the Rfam (Nawrocki et al., 2015), GenBank, and Repbase databases (Kapitonov and Jurka, 2008); then, they were grouped into ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small cytoplasmic RNA (scRNA) (Gardner et al., 2011), mapped sRNA, and unannotated sRNA using Bowtie software (Burke et al., 2013; Langmead et al., 2009; Mishra et al., 2016). After removal of the tRNAs, rRNAs, scRNAs, snRNAs, and snoRNAs, the remaining unique sRNA sequences were used as queries in BLASTn searches against known plant miRNAs in the miRBase database (version 21.0) (Griffiths-Jones et al., 2008) based on the following criteria: 1) the unique sRNA sequences aligned to an miRNA precursor in the miRBase database with no mismatch, and 2) the unique sRNA sequences aligned to mature miRNAs in the miRBase database with an overlap of at least 16 nt, allowing offsets (Meyers et al., 2008). Unidentified sequences were further analyzed to predict novel miRNAs using miRDeep2 software (version 2.0.5) with modified parameters for plant species as previously described (Meyers et al., 2008; Zhang and Wang, 2015). Based on the transcriptome sequences of *P.rockii* and *P.ostii* (NCBI accession no. SRP109687), the conserved and novel miRNA secondary structures were predicted using RNAfold tools (version 2.0) with the default parameters (Chu et al., 2016).

MiRNA expression analysis. To identify differentially expressed miRNAs, IDEG6 software was used (Romualdi et al., 2003). Additionally, an abundance of each identified miRNA was normalized to transcripts per million (TPM) with the following formula: read count mapped to actual miRNA × 1,000,000/total count of clean reads (Mishra et al., 2016). The fold change (FC) between the PR and PO libraries at each developmental stage was calculated as the log2 (TPMs of PR/TPMs of PO). The false discovery rate (FDR) control is a statistical method used for multiple hypothesis testing to correct for the probability value. In this analysis, | log2 (FC) | ≥1 and FDR ≤0.05 were set as the thresholds to denote the significance of differential expression. If the value of log2 (FC) was positive, then upregulation of the miRNA was indicated, whereas a negative value indicated downregulation. Venn diagrams of the differentially expressed miRNAs at the different stages of PO and PR were obtained using Venny software [version 2.1.0 (Li et al., 2018)]. Heatmaps of the miRNA expression levels were generated using Heml software with the value of log2 [TPM (Deng et al., 2014)].

Target gene prediction and functional annotation. The conserved and novel miRNAs of PO and PR were used as query sequences to search for target genes against the PO and PR petal transcriptome data using Target Finder (version 1.6) (Allen et al., 2005), and a score <3.0 was set as the threshold (Schwab et al., 2005). The following criteria were used to identify potential miRNA targets: 1) no more than four mismatches were allowed between the mature miRNA and its potential target site; 2) no more than one mismatch was allowed at nucleotide positions 1–9; 3) no more than two consecutive mismatches were allowed; and 4) no mismatches were allowed at positions 10 and 11 (De Paola et al., 2012; Xie et al., 2010). The functional categories of the target genes were annotated in the clusters of orthologous groups (COG) database using BLAST with a cut-off e-value <1e-5 (Tatusov et al., 2000). Gene ontology (GO) enrichment analysis was performed to further analyze the function of the target genes and visualized using the Blast2go software (Ashburner et al., 2000). Gossip software was used to analyze the Fisher’s exact test calculations and provided robust FDR corrections for multiple testing and a list of significant GO terms ranked according to their corrected probability value (*P* < 0.05), which were used for the GO annotations (Conesa et al., 2005).

Analysis of miRNAs and their target genes by qRT-PCR. Petals from five different flowers at the same opening stage of five PO and PR individuals were combined to form one sample, and three biological replicates were collected for each sample. Petal samples were detached from five different opening stages (S1, S2, S3, S4, and S5) of PO and PR, respectively. Then, these samples were used to extract total RNA using Trizol reagent. The cDNAs were synthesized from the total RNA using M-MLVRT (Promega, Madison, WI) and miRNA-specific stem-loop reverse-transcription (RT) primers. Quantitative real-time polymerase chain reaction (qRT-PCR) experiments were performed using an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster, CA) and a SYBR Premix Ex Taq Kit (Takara, Otsu, Japan) to monitor cDNA amplification using specific forward primers and a universal reverse primer (Supplemental Table 1) according to the manufacturer’s specifications. The reactions were incubated at 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, and 4 °C for 5 min. The cycle conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 43 cycles at 95 °C for 10 s, 58 °C for 20 s, and 72 °C for 10 s. Melting curves were obtained at 95 °C for 5 s and 58 °C for 1 min, followed by cooling to 40 °C for 30 s; these were used to detect the specificity of the amplified product (Unver and Budak, 2009). U6 snRNAs were selected as the internal controls for the miRNAs.

In addition, target gene expression was examined using the same qRT-PCR system. RT reactions were performed using M-MLVRT (Promega) according to the manufacturer’s instructions. The amplification procedure was performed as
follows: 95 °C for 10 min and 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 20 s at 72 °C. All reactions, both biological and technical, were performed in triplicate. TUB was used as a reference gene according to Shi et al. (2017). All primers are listed in Supplemental Table 1. For the data analysis, the expression levels of the miRNAs and target genes were calculated using the $2^{-\Delta\Delta Ct}$ method, and the FCs (PR/PO) of the miRNA and the target gene expression were achieved by calibrating the transcription levels of PR petals at different stages to those of PO petals. Standard deviations were obtained from three biological replicates and three technical replicates. In addition, the correlation coefficient was calculated based on the FCs of miRNA and the target gene expression according to Pearson’s correlation coefficient by SPSS (version 19.0; IBM Corp., Armonk, NY).

**Results**

High-throughput sequencing and characterization of potential miRNAs. Six sRNA libraries of PO and PR petals at three different opening stages were constructed and sequenced. After aligning to known plant miRNAs in the miRBase database (version 21.0), we obtained 2,346,618 (11.59%), 3,167,799 (16.44%), 2,732,355 (17.11%), 3,278,428 (16.15%), 2,850,655 (13.06%), and 2,923,017 (14.21%) mapped miRNA reads and 10,773,418 (53.19%), 12,011,249 (62.35%), 8,141,546 (50.98%), 13,207,659 (65.07%), 11,878,977 (54.42%), and 11,072,082 (53.82%) unannotated sRNA reads from the PO-S1, PO-S3, PO-S5, PR-S1, PR-S3, and PR-S5 libraries, respectively (Supplemental Tables 2 and 3). The large number of unannotated sRNAs suggested that many novel miRNAs exist in tree peony. Most obtained sRNAs ranged from 18 to 30 nt in length (Supplemental Table 3). The mature miRNAs were 21 to 24 nt in length, which is a representative size range for Dicer-derived products, and the most frequent sRNA sequence lengths were 21 and 22 nt (Supplemental Fig. 1, Supplemental Table 3). According to the expression analysis, the median value of the expression quantity of PR was higher than that of PO. In addition, the miRNA expression level was higher during S1 than during the other opening stages (Fig. 2A). Furthermore, the expression differences in the six miRNA libraries indicated that the six samples clustered into two primary clusters. The three PO libraries formed a cluster with PR-S1 and PR-S3, whereas PR-S5 formed a single branch (Fig. 2B), representing the special pigmentation at the fully open stage of PR.

Identification of conserved and novel miRNAs and their expression profiles in PO and PR. The six sRNA datasets of PO and PR were aligned to known miRNA sequences in the miRBase database. In total, we identified 22 conserved miRNAs and 27 novel miRNAs (Tables 1 and 2, Supplemental Fig. 2). The 22 conserved miRNAs belonged to 16 different families, and the highest representation included four miRNAs from the miR396 family, three miRNAs from the miR159 family, and two miRNAs from the miR171_1 family. The remaining 13 miRNA families only had one member. According to the sequencing results, the expression levels of different members from the same miRNA family were highly variable. The expression level of miR159a was ∼10-fold higher than that of miR159c, whereas the expression of miR159b was lower (Table 1). The 27 novel miRNAs were designated novel_miR_01 to novel_miR_27, with correct secondary hairpin structures and hairpin energies ranging from −5.9 to −96.1 kcal/mol (Table 2). Among the 49 miRNAs, six miRNAs were identified only in

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**Fig. 2.** A study of the overall expression patterns of all miRNAs in the six miRNA libraries. (A) General distribution of the log_{10}-transformed expression values [transcripts per million (TPM)] of all miRNAs in the six libraries. (B) Correlations among the six miRNA libraries based on the Pearson correlation coefficient. The color scale represents the Pearson correlation coefficients among the different samples. The higher the Pearson correlation coefficient, the closer the relationship between the two libraries. Blue represents a close relationship; pink represents a distant relationship.
Table 1. Expression profiles and sequences of conserved miRNAs in petals of *Paeonia rockii* and *Paeonia ostii* at different opening stages.

| miRNA Accession | miRNA Family | Mature sequence | Hairpin energy (kcal/mol) | Transcriptions (no./million) | Fold-change PR vs. PO | Fold-change PR vs. PR | Fold-change PO vs. PO |
|-----------------|--------------|-----------------|--------------------------|-----------------------------|-----------------------|----------------------|-----------------------|
| c120506.graph\_c0_15452 | miR159a | uauuggaggaagggagcucua | -102 | 821,199.90 | 722,361.70 | 798,076.00 | 763,178.20 | 776,819.60 | 677,528.20 | -0.11 | 0.11 | -0.24 | 0.03 | -0.20 | -0.19 | 0.14 |
| c113537.graph\_c0_4962 | miR159b | uauuggaggaagggagcucua | -69.2 | 9,446.66 | 7,468.87 | 4,068.32 | 14,029.29 | 12,318.00 | 7,954.72 | 0.57 | 0.72 | -0.19 | -0.37 | -0.34 | -0.87 |
| c113537.graph\_c0_4959 | miR159c | uauuggaggaagggagcucua | -87.5 | 326.98 | 750.03 | 1,081.54 | 1,498.55 | 1,718.16 | 2,613.93 | 2.20 | 1.20 | -0.24 | -0.20 | -0.19 | 0.14 |
| c50248.graph\_c0_32962 | miR162_2 | uauuggaggaagggagcucua | -68.9 | 5,560.03 | 5,240.00 | 1,000.00 | 1,400.00 | 1,700.00 | 2,600.00 | 2.20 | 1.20 | -0.24 | -0.20 | -0.19 | 0.14 |
| c105628.graph\_c0_1828 | miR168 | uauuggaggaagggagcucua | -61.1 | 7,430.28 | 9,351.14 | 8,392.91 | 4,171.90 | 4,674.48 | 7,298.23 | -0.83 | -1.00 | -0.20 | 0.16 | 0.64 | -0.16 |
| c137761.graph\_c0_26900 | miR171_1a | uauuggaggaagggagcucua | -73.1 | 1,030.33 | 1,250.05 | 1,067.81 | 1,090.10 | 1,225.44 | 1,164.46 | 1.32 | 0.24 | 1.96 | -0.13 | 0.73 | -0.98 |
| c122708.graph\_c0_10537 | miR171_1b | uauuggaggaagggagcucua | -73.1 | 260.56 | 501.33 | 253.91 | 650.24 | 592.72 | 985.31 | 1.32 | 0.24 | 1.96 | -0.13 | 0.73 | -0.98 |
| c68622.graph\_c0_34805 | miR319 | uauuggaggaagggagcucua | -64.4 | 1,415.21 | 157.07 | 215.48 | 315.56 | 330.90 | 580.19 | -2.17 | 1.07 | 1.43 | 0.07 | 0.81 | -3.17 | 0.46 |
| c118957.graph\_c0_7979 | miR390 | uauuggaggaagggagcucua | -67.7 | 556.89 | 166.24 | 561.36 | 1,030.00 | 344.24 | 372.72 | 460.08 | -0.69 | 1.16 | 1.29 | 0.11 | 0.30 | -1.74 | 1.76 |
| c130103.graph\_c0_18018 | miR396a | uauuggaggaagggagcucua | -54.7 | 26,795.32 | 49,660.26 | 34,915.34 | 46,058.96 | 36,930.37 | 78,077.83 | 0.78 | -0.43 | 1.16 | -0.32 | 1.08 | 0.89 | -0.51 |
| c103359.graph\_c0_1151 | miR396b | uauuggaggaagggagcucua | -34.2 | 2,191.79 | 26,980.08 | 10,922.45 | 12,100.43 | 7,052.63 | 19,258.37 | 2.46 | -1.94 | 0.82 | -0.78 | 1.45 | 3.62 | -1.30 |
| c103707.graph\_c0_34299 | miR396c | uauuggaggaagggagcucua | -73.8 | 2,835.53 | 15,508.44 | 7,694.30 | 12,242.50 | 7,052.63 | 19,258.37 | 2.46 | -1.94 | 0.82 | -0.78 | 1.45 | 3.62 | -1.30 |
| c186826.graph\_c0_26929 | miR398 | uauuggaggaagggagcucua | -35.9 | 40.87 | 99.48 | 85.10 | 61.47 | 116.36 | 118.07 | 0.59 | 0.23 | 0.47 | 0.92 | 0.02 | 1.28 | -0.23 |
| c49039.graph\_c0_32846 | miR4221 | uauuggaggaagggagcucua | -96.5 | 12,506.98 | 11,178.43 | 7,946.85 | 4,068.32 | 14,029.29 | 12,318.00 | 7,954.72 | 0.57 | 0.72 | -0.19 | -0.37 | -0.34 | -0.87 |
| c130103.graph\_c0_18018 | miR5267 | uauuggaggaagggagcucua | -16.3 | 13,925.60 | 11,677.14 | 13,285.92 | 3,057.21 | 18,274.28 | 10,779.39 | -2.19 | 0.65 | -0.30 | 2.58 | -0.76 | -0.25 | 0.19 |
| c95348.graph\_c0_39372 | miR530 | uauuggaggaagggagcucua | -64.6 | 61.31 | 319.38 | 171.56 | 1,030.00 | 892.71 | 2,155.88 | 4.07 | 1.48 | 3.65 | -0.21 | 1.27 | 2.38 | -0.90 |
| c102029.graph\_c0_8729 | miR5303 | uauuggaggaagggagcucua | -72.5 | 246.94 | 102.10 | 94.70 | 180.32 | 158.18 | 496.73 | -0.45 | 0.63 | 2.39 | -0.19 | 1.65 | -1.27 | -0.11 |
| c124819.graph\_c0_12229 | miR5304 | uauuggaggaagggagcucua | -72.5 | 129.43 | 154.46 | 64.51 | 486.31 | 399.99 | 531.34 | 1.91 | 1.37 | 3.04 | -0.28 | 0.41 | 0.26 | -1.26 |

The miRNA accession no. could be obtained from small RNA sequences of *P. rockii* (PR) and *P. ostii* (PO) with NCBI accession no. SRP102723. PO-S1, PO-S3, and PO-S5 are *P. ostii* petals at three different opening stages (S1, S3, and S5, respectively). PR-S1, PR-S3, and PR-S5 are *P. rockii* petals at three different opening stages (S1, S3, and S5, respectively).
Table 2. Expression profiles and sequences of novel miRNAs in petals of *Paeonia rockii* and *Paeonia ostii* at different opening stages.

| miRNA Accession | miRNA Family | Mature sequence | Hairpin energy (kcal mol⁻¹) | Transcriptions (no./million) | Fold change | PR vs. PO | PR vs. PO | PO vs. PO |
|----------------|--------------|----------------|-----------------------------|-----------------------------|------------|----------|----------|----------|
| c133439.graph.c0_25698 | novel_mir_01 | ccaacgugucucaagauugucg | -50.8 | 16,687.90, 14,863.13, 14,062.76, 15,006.01, 17,028.84, 27,743.45 | 0.15, 0.20, 0.98, 1.02, 0.18, 0.70 | 0.17, 0.08 |
| c139609.graph.c0_27008 | novel_mir_02 | caaggucauauauauaugga | -14.5 | 0.00, 0.00, 0.00, 62.84, 36.36, 22.39 | -0.79, -0.70 | -0.08 |
| c133751.graph.c1_23627 | novel_mir_03 | ccuacuauaagauugugaug | -5.9 | 49.39, 155.77, 109.80, 2,088.68, 416.36, 1,691.72 | 5.40, 1.42, 3.95 | 2.02, 1.66 |
| c115112.graph.c1_5756 | novel_mir_04 | uugacagaagagagggagcac | -74.6 | 625.01, 2,088.68, 892.03, 585.45, 639.23 | 3.78, -5.27, -3.97 | 6.93, 5.08 |
| c107606.graph.c0_2588 | novel_mir_05 | auuucuacuacuccaucuacg | -71.5 | 182.22, 6,155.99, 14,197.27, 2,499.86, 19,459.91 | 0.51, 1.15, 2.36 | -0.61, 1.07 |
| c129372.graph.c0_17104 | novel_mir_06 | uacagucuacucuauau | -12.3 | 245.23, 229.21, 9.56, 40.72 | -4.68, -3.53, 1.93 | 0.08, 0.58 |
| c133345.graph.c0_27974 | novel_mir_07 | aagauauaggcguaaaaugaga | -55.2 | 241.83, 142.68, 101.57, 202.17, 183.63, 168.97 | -0.26, 0.36, 0.73 | -0.14, 0.12 |
| c123969.graph.c0_11466 | novel_mir_08 | ugaacgagagaagacagac | -78.6 | 904.30, 227.76, 229.21, 3,087.26, 1,045.44, 187.29 | 1.77, 2.20, -0.29 | -1.56, -2.48 |
| c115067.graph.c0_5737 | novel_mir_09 | aagaacgcaagacgacaa | -57.8 | 28.95, 18.33, 15.10, 43.71, 70.91, 38.68 | 0.59, 1.95, 1.36 | 0.70, -0.66, -0.28 |
| c126232.graph.c0_13567 | novel_mir_10 | uguuugucuacuagcugu | -44.8 | 54.50, 45.81, 46.67, 16.39, 72.73, 95.68 | -1.73, 0.67, 1.04 | 2.15, 0.40, -0.25 |
| c82394.graph.c0_32983 | novel_mir_11 | uaugugucucgcuuagc | -63.2 | 15.33, 27.49, 20.59, 0.00, 0.00, 0.00 | --- | --- | --- |
| c134424.graph.c0_26202 | novel_mir_12 | uccccagcuaagacucu | -51.4 | 5.11, 3.93, 8.24, 0.00, 5.45, 0.00 | --- | 0.47, --- | --- |
| c143351.graph.c0_27209 | novel_mir_13 | ccaugucuacucgucuagau | -106.6 | 15.33, 18.33, 30.20, 1.37, 0.00, 2.04 | -3.49, --- | -3.89, --- | --- |
| c50127.graph.c0_32983 | novel_mir_14 | uuaacgugucuacuucucu | -62.3 | 408.72, 407.08, 310.19, 0.00, 0.00, 0.00 | --- | --- | --- |
| c87345.graph.c0_37619 | novel_mir_15 | ggaaguguguagaa | -33.5 | 22.14, 45.81, 32.94, 0.00, 0.00, 0.00 | --- | --- | --- |
| c66332.graph.c0_34524 | novel_mir_16 | uuggcuauguaucuagc | -75 | 1,578.70, 1,723.89, 951.15, 2,872.79, 4,321.76, 1,581.79, 0.86, 1.33, 0.73, 0.59, -1.45 | 0.13, -0.86 |
| c115909.graph.c0_6259 | novel_mir_17 | aacuucuacuccucua | -66.8 | 415.54, 670.18, 317.05, 0.00, 0.00, 0.00 | --- | --- | --- |
| c100429.graph.c0_14368 | novel_mir_18 | uccagucuacuagcuu | -6.3 | 0.00, 0.00, 0.00, 183.05, 3.64, 582.23 | --- | --- | --- |
| c111239.graph.c0_9899 | novel_mir_19 | uucuucuacuucuagau | -6.1 | 0.00, 0.00, 0.00, 95.62, 58.18, 69.22 | --- | --- | --- |
| c127131.graph.c0_14368 | novel_mir_20 | ugcagugauagauggaga | -75.6 | 207.77, 535.36, 811.15, 87.43, 163.63, 891.67, -1.25, -1.71, 0.14 | 0.90, 2.45, 1.37, 0.60 |
| c36563.graph.c0_31798 | novel_mir_21 | uguuguacuauauauag | -25.1 | 45.98, 62.83, 59.02, 265.01, 96.36, 136.40, 2.53, 0.02, 0.12, 1.46 | 0.50, 0.45, -0.09 |
| c123399.graph.c0_11078 | novel_mir_22 | uaaucuacuagcuaagggca | -73.2 | 21,395.05, 28,142.43, 24,771.10, 21,741.98, 14,523.43, 26,082.27 | 0.02, -0.95, 0.07, -0.58, 0.84, 0.40, -0.18 | 0.17, 0.08 | --- | --- |

Continued next page
Table 2. Continued.

| miRNA Accession | Transcriptions (no./million) | Fold change (PR vs. PO) | PR vs. PO | PO vs. PO | S1 | S3 | S5 |
|-----------------|------------------------------|------------------------|-----------|-----------|----|----|----|
| c126048.graph    | 2.306.53                     | 1.049.06               | 1.087.74  | 863.31    | -97.3 | 221.4 | 32.72 |
| c0_13401         | 18.73                        | 4.12                   | 4.10      | 12.73     | 1.70 | 2.62 | 4.12 |
| c120720.graph    | 43.64                        | 34.61                  | 34.61     | 14.25     | 1.70 | 2.62 | 4.12 |
| c0_9027          | 14.25                        | 12.73                  | 12.73     | 1.70      | 2.62 | 4.12 | 4.12 |
| c126494.graph    | 0.16                         | 0.65                   | 0.62      | 0.62      | 0.62 | 0.62 | 0.62 |

The miRNA accession no. could be obtained from small RNA sequences of *P. rockii* (PR) and *P. ostii* (PO) with NCBI accession number SRP102723. miRNA energy (kcal/mol–1) was calculated using RNAhybrid software. The mature site sequences were obtained from the GeneBank (accession no. EF524693) database. The putative targets were predicted using Target Finder software (version 1.6). In this study, we obtained 33 and 61 targets for 19 conserved and 20 novel miRNAs, respectively (Supplemental Table 5). More than one unigene were identified as candidate targets for most miRNAs. Based on the COG analysis, the putative targets of miRNAs that showed higher expression levels in PR were involved in transcription, general function prediction only, secondary metabolites biosynthesis, transport and catabolism, and signal transduction mechanisms, whereas those in PO were involved in chromatin structure and dynamics, replication, recombination and repair, and defense mechanisms (Supplemental Table 5). Moreover, the target genes of miRNAs that were highly significantly expressed during variegation formation in PR petals, such as miR396a, miR396b, miR396d, miR530, miR5303, novel_miR_03, novel_miR_04, novel_miR_18, and novel_miR_21, were downregulated at PR-S3 but upregulated at PR-S5.

**Target prediction analysis of miRNAs.** To better understand the functions of the conserved and novel miRNAs in flowers of tree peony, the putative targets were predicted using PR and PO petal transcriptome data (NCBI accession no. SRP109687) and Target Finder software (version 1.6). In this study, we obtained 33 and 61 targets for 19 conserved and 20 novel miRNAs, respectively (Supplemental Table 5). More than one unigene were identified as candidate targets for most miRNAs. Based on the COG analysis, the putative targets of miRNAs that showed higher expression levels in PR were involved in transcription, general function prediction only, secondary metabolites biosynthesis, transport and catabolism, and signal transduction mechanisms, whereas those in PO were involved in chromatin structure and dynamics, replication, recombination and repair, and defense mechanisms (Supplemental Table 5). Moreover, the target genes of miRNAs that were highly significantly expressed during variegation formation in PR petals, such as miR396a, miR396b, miR396d, and novel_miR_03, were annotated as inorganic ion transport and metabolism and transportation. In addition, the GO categories of
the targets of these differentially expressed miRNAs were abundant in the molecular function categories “binding,” “ATP binding,” and “nucleotide binding,” and in the biological category “metabolic process” (Supplemental Table 5).

Accordingly, these predicted targets belonged to several TFs and functional gene families with various biological functions. Five MYB TFs were found to be putative target genes of miR159c, miR168, miR319, and novel_miR_05, of which the putative targets of miR159c and novel_miR_05 were annotated as the anthocyanin regulators ZmC1 and LiPAP, respectively. Four bHLH TFs were predicted to be targets of miR159 and miR396. Additionally, many functional genes were predicted to be targets of miRNAs such as CHS and ABC transporter. miR168 also targeted the CHS gene in PR and PO petals. The ATP-binding glutathione S-transporter and ATPase family proteins were considered to be targets of miR7492, miR396, and novel_miR_19 (Supplemental Table 5).

**QRT-PCR ANALYSES OF miRNAs AND THEIR TARGET GENES.** Based on...
the predicted target genes, we analyzed the expression levels and corresponding FCs of six miRNAs (five conserved and one novel miRNA) and their predicted target genes that were involved in anthocyanin biosynthesis during PR and PO flower opening by qRT-PCR (Fig. 5, Supplemental Table 5). In relation to PO, the expressions of miR159c, miR396a, miR396b, and novel_miR_05 were considerably up-regulated during most PR opening stages (Fig. 5A, D, E, F, G, and H), whereas miR168 was down-regulated throughout PR petal pigmentation (Fig. 5B and I). During PR spot formation, miR159c and novel_miR_05 increased until S3, decreased at S4, and was upregulated at S5 (Fig. 5A, D, and E), and the expression of miR168 slightly increased from S1 to S5 (Fig. 5B and H).

Validated plant miRNA targets are highly complementary to their respective miRNAs (Yin et al., 2016). Certain targets of the six miRNAs exhibited higher expression levels in PR than in PO except for the miR159c target c115438.graph_c0 (MYB1) at S5, the miR319 target c194399.graph_c0 (MYB3) from S3 to S5, and the miR396a target c130130.graph_c0 (bHLH1) at S2 and S3 (Fig. 5). In comparison with PO during petal pigmentation, c115438.graph_c0 (MYB1) and c119993.graph_c0 (MYB3) showed similar expression patterns; the highest expression levels were observed at S1, whereas the lowest levels were observed at S3 (Fig. 5A and E). In relation to the petal pigmentation of PO, miR319 exhibited a continuous increase, whereas its target c194399.graph_c0 (MYB3) displayed a...
continually decreasing trend (Fig. 5C). During PR petal opening, expressions of c105628.graph_c0 (MYB2), c103359.graph_c0 (bHLH2), and c129851.graph_c0 (ATP-binding glutathione S-conjugate) displayed a sharp increase from S1 to S3, and then a sharp decrease from S3 to S5 (Fig. 5I). CHS, the target of miR168, exhibited a persistent decreasing expression pattern, relative to PO, during PR petal opening (Fig. 5I). In the present study, the number of miRNAs was similar to that in genome sequences, have been reported (Chu et al., 2016). In the present study, the number of miRNAs was similar to that in genome sequences, have been reported (Chu et al., 2016). Accordingly, 22 known and 27 novel miRNAs were mapped to cross species is possible (Roy et al., 2016). Therefore, the targets of miR159c and novel_miR_05 showed a significant negative correlation with these miRNAs in pigmentation. Based on the target analysis and the expression data of miRNAs and targets, miR159c, miR168, miR396a, and novel_miR_05 were predicted to be involved in variegation formation in P. rockii.

Discussion

Paeonia rockii is a wild tree peony species with wide, deep purple variegation at the base of the petals, whereas P. ostii displays purely white petals. miRNAs regulate plant development and morphogenic processes. In this study, we first constructed sRNA libraries of petals from P. rockii and P. ostii at different flower opening stages and identified sRNAs involved in variegation formation in petals of P. rockii. This work provides further insight into the regulatory mechanisms of miRNAs in pigmentation.

Several miRNAs of different plant species, most with genome sequences, have been reported (Chu et al., 2016). In the present study, the number of miRNAs was similar to that in the P. ostii ‘Feng dan’ flower bud (Zhang et al., 2018); however, it was much greater than that reported for seed libraries of tree peony (Yin et al., 2018), and it was much less than that reported for miRNAs involved in flower coloration in P. lactiflora (Zhao et al., 2015, 2017). The reason why so few miRNAs were mapped might be the lack of tree peony genome sequences and the limited number of transcript data in public databases (Zhang et al., 2018). Although the approach of identifying miRNAs by using comparisons with transcriptome databases or genome databases of other species of the taxonomically nearest taxa has been followed for organisms without genome sequences, this strategy may underestimate the actual numbers of miRNAs (Dong et al., 2013; Roy et al., 2016).

Because miRNAs are highly conserved among species, the mapping of miRNAs to cross species is possible (Roy et al., 2016). Accordingly, 22 known and 27 novel miRNAs were identified in the petals of the two species, of which six miRNAs were only identified in PO and three miRNAs were only identified in PR. These results may correspond with the nearer taxonomic relationship between PO and PR (Zhou et al., 2014). As expected, the miRNA expression levels in the six libraries corresponded to morphological characteristics during flower development and petal coloration patterns.

Numerous studies have demonstrated that a group of well-conserved miRNAs, including miR156, miR159, miR167, miR169, miR171, miR172, miR319, and miR396, often retain homologous target interactions and perform similar molecular functions across the plant kingdom throughout the course of adaptation and evolution (Axtell et al., 2007). These evolutionarily conserved miRNAs regulate target genes, including SPL, MYB, NAC, HD-ZIPIII, ARF, NF-Y, SCL, AP2, and TCP, which encode transcription factors involved in various metabolic processes, such as growth and development and cellular adaptive responses to adverse circumstances. In the current study, among the putative target genes of the miR396 family, two bHLH TFs (c130130.graph_c0 and c103359.graph_c0) and the ATP-binding glutathione S-conjugate are ABC transporters that has been reported to transport endogenous substances, including herbicides and anthocyanins (Lu et al., 1997). Here, the target gene of miR396a, ATP-binding glutathione S-conjugate was a higher expression level in PR than in PO, indicating that the crosstalk between miR396a and ATP-binding glutathione S-conjugate may perform critical functions in anthocyanin transport and, consequently, flower pigmentation. Furthermore, miR159, miR168, and miR319 are well-conserved miRNAs in plants and have overlapping roles in the regulation of flavonoid biosynthesis pathways and flower development (Hong and Jackson, 2015; Roy et al., 2016). Among these, miR159c and novel_miR_05 target R2R3-MYB regulators of anthocyanin biosynthesis, which are homologous with the MYB TFs involved in flower coloration regulation in Zea mays and Lotus japonicas, respectively (Wang et al., 2016; Yoshida et al., 2010; Zong et al., 2017), and highly homologous with PsMYB12 in tree peony (Gu et al., 2018). Moreover, two R2R3-MYBs were expressed more highly in PR. Similar to the representative tree peony cultivar Qing Hai Yin Bo, PsMYB12 and PsCHS were expressed more intensely in the blotches than in the nonblotch areas when the spot formed (Gu et al., 2018). Additionally, in Oncidium ‘Gower Ramsey’ and Lilium species, the differentially expressed gene OgMYB1 is actively expressed in red spots and determines the color pattern of floral organs (Chiou and Yeh, 2008). High expression of LhMYB18 is linked to large spot formation in tepals of Lilium species (Yamagishi, 2018), similar to the Phalaenopsis species. Among the three R2R3-MYB TFs involved in the regulation of anthocyanin biosynthesis, PeMYB11 is responsive to the red spots in the calyx of the lip, but it is slightly expressed in the lip (Hsu et al., 2015). Correspondingly, the FCs of miR159c and novel_miR_05 showed a significant negative correlation with these two R2R3-MYB TFs. Therefore, the targets of miR159c and novel_miR_05 are involved in the pigmentation of PR petals by triggering structural genes before variegation formation. In addition, the lower expression level of miR168 in PR might down-regulate the early steps of the flavonoid biosynthesis pathway. Its target CHS, which is the first key enzyme in the flavonoid biosynthesis pathway, was higher in PR than in PO. This result was in agreement with those of previous reports for the tree peony cultivar Qing Hai Yin Bo and C. indica (Gu et al., 2018; Roy et al., 2016). Therefore, we inferred that miR159c, miR168, miR396a, novel_miR_05, and their corresponding target genes may be associated with anthocyanin accumulation,
thereby influencing pigmentation at the base of the petals in *P. rockii*. Nevertheless, the mechanisms of regulation of variegation formation and flower pigmentation are so combinatorial that further exploration of the differentially expressed mir159c, mir168, mir396a, novel_mir_05, and their corresponding targets (MYB TFs, CHS, and ABC transporters) is necessary to determine the miRNA-mediated regulatory mechanisms of variegation formation in *P. rockii*.

In conclusion, this is the first report of miRNAs in the petals of the nonmodel plant tree peony using Illumina HiSeq sequencing technology. In total, 49 miRNAs were identified, of which 40 were differentially expressed during flower development. In addition, we further characterized potential candidate target genes encoding key enzymes involved in the anthocyanin biosynthesis pathway in *P. rockii*. The differential expressions of mir159c, mir168, mir396a, and novel_mir_05 were associated with targets encoding MYBs, CHS, and ABC transporters, which might participate in the variegated pigmentation in *P. rockii*. Our results provide valuable resources and a substantial basis for elucidating the molecular mechanisms controlling petal variegation in *P. rockii*.

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Supplemental Fig. 1. The study of the length distribution of miRNAs identified in *Paeonia rockii* and *Paeonia ostii* petals. The x-axis represents the length of miRNAs. The y-axis represents the number of miRNAs with a specific length.
Supplemental Fig. 2. Predicted precursor structures of all identified miRNAs. (A) Conserved miRNAs. (B) Novel miRNAs. Red sequences represent the mature sequences. Yellow sequences represent the loop structure. Purple sequences represent the star sequences predicted by miRDeep2 software. Blue sequences represent the star sequences from reads by sRNA sequencing.
Supplemental Fig. 3. The study of correlations between the gene expression results obtained by qRT-PCR analysis and those obtained using Illumina sequencing for the 32 identified miRNAs at three different opening stages (S1, S3, and S5) in *Paeonia ostii* and *P. rockii* petals. The correlations were analyzed based on the average log2 values of expression levels of each miRNA during the three opening stages of *P. ostii* and *P. rockii* (S1 = unpigmented tight bud; S2 = slightly soft bud without pigmentation; S3 = initially open flower with slight pigmentation; S4 = half-open flower with slight pigmentation; S5 = fully open and pigmented flower with exposed anthers).
**Supplemental Table 1. Sequences of primers used in this study for qRT-PCR.**

| Gene name | Target gene | Forward (5′ to 3′) | Reverse (5′ to 3′) |
|-----------|-------------|--------------------|--------------------|
| MYB1      | c115438.graph_c0 | TGCTGGATGCTTGCTTACAC | TTACTCTCGGGAATTCACC |
| MYB2      | c105628.graph_c0 | AAACCTTACACTGACTGCTG | TCAGTCGTTTTATTGGTTTG |
| MYB3      | c194399.graph_c0 | GCTGACATGGCAAGAGTA | GGCTTTGCTTTTCATTCAG |
| MYB4      | c121983.graph_c0 | TGGAGCAGTGGTCAGCGGA | TAAACCACACATGGCAACAG |
| bHLH1     | c130130.graph_c0 | TTTTCCTTCTTTCCTTCCC | ATCAGGCTGTTCCTCCACAG |
| bHLH2     | c103539.graph_c0 | ATGCAAGTGGCAGCTTTTG | ATTCAGGCTGTTCCTCCAG |
| ATP-binding Glutathione S-conjugate | | | |
| CHS       | c43510.graph_c0 | CGCAATCTCTACACAG | CATGAGCGTTCTGGATTG |
| TUB       | c106431.graph_c0 | AGGTAAGATGAGCACAAAG | GGAAGGAGATGTCACAAAGC |
| miRNA Family | miRNAs | Stem-loop RT primer (5′ to 3′) | Forward (5′ to 3′) |
| miR159a   | c128056.graph_c0_15452 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR159b   | c11357.graph_c0_4962 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR159c   | c11357.graph_c0_4962 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR162_2  | c50248.graph_c0_3296 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR168    | c105628.graph_c0_1828 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR171_1b | c122708.graph_c0_10537 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR319    | c68622.graph_c0_34805 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR390    | c118957.graph_c0_7979 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR396a   | c130130.graph_c0_18018 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR396b   | c103359.graph_c0_1151 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR396c   | c64070.graph_c0_34299 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR396d   | c103359.graph_c0_1152 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR398    | c186826.graph_c0_29629 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR4221   | c49039.graph_c0_32846 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR5079   | c95284.graph_c0_39372 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR530    | c92818.graph_c0_38794 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR5303   | c89765.graph_c0_38143 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR7492   | c124819.graph_c0_12229 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| miR827_4  | c98608.graph_c0_40285 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| novel_miR_03 | c133751.graph_c1_23627 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| novel_miR_04 | c115112.graph_c0_5756 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| novel_miR_05 | c107606.graph_c0_2588 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| novel_miR_06 | c29372.graph_c0_17104 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| novel_miR_08 | c123969.graph_c0_11468 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| novel_miR_09 | c115067.graph_c0_5737 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| novel_miR_10 | c126232.graph_c0_13567 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| novel_miR_17 | c115907.graph_c0_6259 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| novel_miR_19 | c111239.graph_c0_3989 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| novel_miR_20 | c127131.graph_c0_14368 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| novel_miR_21 | c36563.graph_c0_31798 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| novel_miR_24 | c113180.graph_c0_4822 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| novel_miR_27 | c120720.graph_c0_9027 | GTCGTATCCAGTGGATTCGCACTGGATACGACTGGAGC | GCGCGCTTTGAGTGGAGGAG |
| U6 snRNAs | — | — | — |
| Universal reverse primers | — | — | — |
## Supplemental Table 2. Distribution of small RNA reads among different RNA categories in *Paeonia rockii* and *P. ostii* petals at three different opening stages.

| Type       | PO-S1  | PO-S3  | PO-S5  | PR-S1  | PR-S3  | PR-S5  |
|------------|--------|--------|--------|--------|--------|--------|
| Total clean reads | 20,253,112 | 100.00 | 19,264,038 | 100.00 | 15,969,634 | 100.00 | 20,298,607 | 100.00 | 21,827,267 | 100.00 | 20,574,106 | 100.00 |
| rRNA       | 6,925,449 34.19 | 3,926,506 20.38 | 4,886,135 30.60 | 3,657,285 18.02 | 6,832,305 31.30 | 6,388,857 31.05 |
| scRNA      | 0 0.00 0.00 | 0 0.00 0.00 | 0 0.00 0.00 | 0 0.00 0.00 | 0 0.00 0.00 | 0 0.00 0.00 |
| snoRNA     | 5,519 0.03 | 3,076 0.02 | 2,697 0.02 | 3,161 0.02 | 4,689 0.02 | 3,335 0.02 |
| tRNA       | 168,100 0.83 | 128,182 0.67 | 174,716 1.09 | 128,307 0.63 | 231,399 1.06 | 159,992 0.78 |
| Repbase    | 34,007 0.17 | 27,226 0.14 | 32,183 0.20 | 23,766 0.12 | 29,242 0.13 | 26,823 0.13 |
| Unannotated | 10,773,418 53.19 | 12,011,249 62.35 | 8,141,546 50.98 | 13,207,659 65.07 | 11,878,977 54.42 | 11,072,082 53.82 |
| Mapped_Reads | 2,346,618 11.59 | 3,167,799 16.44 | 2,732,355 17.11 | 3,278,428 16.15 | 2,850,655 13.06 | 2,923,017 14.21 |
| Mapped_reads (+) | 1,026,849 0.050700801 | 1,547,653 0.080338972 | 1,271,583 0.079625056 | 1,485,157 0.073165464 | 1,301,071 0.0596076 | 1,495,446 0.072685831 |
| Mapped_reads (–) | 1,319,769 6.52 | 1,620,146 8.41 | 1,460,772 9.15 | 1,793,271 8.83 | 1,549,584 7.10 | 1,427,571 6.94 |

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*PO-S1, PO-S3, and PO-S5 are *P. ostii* (PO) petals at three different opening stages (S1, S3, and S5, respectively). S1 = unpigmented tight bud. S2 = slightly soft bud without pigmentation. S3 = initially open flower with slight pigmentation. S4 = half-open flower with slight pigmentation. S5 = fully open and pigmented flower with exposed anthers.*

*PR-S1, PR-S3, and PR-S5 are *P. rockii* (PR) petals at three different opening stages (S1, S3, and S5, respectively).*

*RNA.*
Supplemental Table 3. Length distribution of small RNAs in six libraries derived from *Paeonia rockii* and *Paeonia ostii* petals at three different opening stages.

| Length (nt) | Clean reads (no.) | Mapped reads % | Clean reads (no.) | Mapped reads % | Clean reads (no.) | Mapped reads % | Clean reads (no.) | Mapped reads % | Clean reads (no.) | Mapped reads % |
|------------|-------------------|----------------|-------------------|----------------|-------------------|----------------|-------------------|----------------|-------------------|----------------|
| 18         | 799,186           | 45,399         | 5.68              | 761,909        | 64,961            | 8.53           | 1,120,079         | 68,797         | 6.14              | 592,467         | 45,171          | 7.62           |
| 19         | 1,079,007         | 83,278         | 6.04              | 1,402,428      | 124,812           | 8.90           | 1,609,894         | 130,377        | 8.10              | 1,091,628        | 90,697          | 8.31           |
| 20         | 1,473,711         | 177,513        | 12.05             | 1,491,247      | 249,221           | 16.71          | 1,475,090         | 303,664        | 20.59             | 1,102,002        | 221,533         | 20.10          |
| 21         | 4,149,022         | 1,120,097      | 27.00             | 5,197,304      | 1,700,131         | 32.71          | 4,249,290         | 1,403,287      | 33.02             | 5,038,161        | 1,714,516       | 34.03          |
| 22         | 2,790,506         | 417,028        | 14.94             | 2,878,292      | 528,381           | 18.36          | 2,327,922         | 432,758        | 18.59             | 3,198,622        | 602,522         | 18.84          |
| 23         | 1,628,802         | 118,882        | 7.30              | 1,277,223      | 128,320           | 10.05          | 1,030,334         | 103,055        | 10.00             | 1,506,045        | 145,534         | 9.67           |
| 24         | 5,019,981         | 257,600        | 5.13              | 4,759,588      | 280,283           | 5.89           | 2,853,962         | 211,489        | 7.41              | 6,048,383        | 350,410         | 5.79           |
| 25         | 894,686           | 34,441         | 3.85              | 511,761        | 24,410            | 4.77           | 397,587           | 21,268         | 5.35              | 618,272          | 35,002          | 5.66           |
| 26         | 636,342           | 36,640         | 5.76              | 309,186        | 34,883            | 11.28          | 260,364           | 24,158         | 9.28              | 345,432          | 31,530          | 9.13           |
| 27         | 502,184           | 21,865         | 4.35              | 208,417        | 12,807            | 6.14           | 186,057           | 12,034         | 6.47              | 244,245          | 14,926          | 6.11           |
| 28         | 402,505           | 14,710         | 3.65              | 187,056        | 8,441             | 4.51           | 165,980           | 8,791          | 5.30              | 209,330          | 11,554          | 5.52           |
| 29         | 325,668           | 10,765         | 3.31              | 150,283        | 6,303             | 4.19           | 147,546           | 7,238          | 4.91              | 166,824          | 8,500           | 5.10           |
| 30         | 251,512           | 8,400          | 3.34              | 129,344        | 4,846             | 3.74           | 145,529           | 5,439          | 3.74              | 137,596          | 6,533           | 4.75           |

| 20,253,112 | 2,346,618         | 11.59          | 19,264,038        | 3,167,299       | 16.44             | 15,969,634      | 2,732,355        | 17.11             | 20,298,607      | 3,278,428       | 16.15          |
| 21,827,267 | 2,850,655         | 13.06          | 20,574,106        | 2,923,017       | 14.21             |

\(^aPO-S1, PO-S3, and PO-S5 are *P. ostii* (PO) petals at three different opening stages (S1, S3, and S5, respectively). S1 = unpigmented tight bud; S2 = slightly soft bud without pigmentation; S3 = initially open flower with slight pigmentation; S4 = half-open flower with slight pigmentation; S5 = fully open and pigmented flower with exposed anthers.\)

\(^bPR-S1, PR-S3, and PR-S5 are *P. rockii* (PR) petals at three different opening stages (S1, S3, and S5, respectively).\)
Supplemental Table 4. The number of differentially expressed and up-regulated miRNAs.

|                      | Conserved miRNAs (no.) | Novel miRNAs (no.) |
|----------------------|------------------------|--------------------|
|                      | Total | Up-regulated | Total | Up-regulated | Total (no.) |
| PR-S1\(^a\) vs. PO-S1\(^a\) | 12    | 8          | 17    | 9            | 29          |
| PR-S3 vs. PO-S3      | 12    | 6          | 17    | 10           | 29          |
| PR-S5 vs. PO-S5      | 13    | 10         | 18    | 10           | 31          |
| PO-S3 vs. PO-S1      | 2     | 2          | 9     | 4            | 11          |
| PO-S5 vs. PO-S3      | 6     | 6          | 6     | 4            | 12          |
| PR-S3 vs. PR-S1      | 11    | 6          | 6     | 4            | 17          |
| PR-S5 vs. PR-S3      | 8     | 3          | 5     | 2            | 13          |

\(^a\)PR-S1, PR-S3, and PR-S5 are *P. rockii* (PR) petals at three different opening stages (S1, S3, and S5, respectively). S1 = unpigmented tight bud; S2 = slightly soft bud without pigmentation; S3 = initially open flower with slight pigmentation; S4 = half-open flower with slight pigmentation; S5 = fully open and pigmented flower with exposed anthers.

\(^b\)PO-S1, PO-S3, and PO-S5 are *P. ostii* (PO) petals at three different opening stages (S1, S3, and S5, respectively).
| miRNA     | Target Accession no. | COG class annotation | GO annotation | Nr annotation |
|-----------|----------------------|-----------------------|---------------|---------------|
| c13537.graph_c0 | miR59a | — | Molecular function: DNA binding (GO:0003677); Biological process: DNA metabolic process (GO:0006259) |
| c114935.graph_c0 | — | — | — | 2-methylene-furan-3-one reductase |
| c125551.graph_c0 | — | — | Molecular function: guanylate kinase activity (GO:0004385); Biological process: purine nucleotide metabolic process (GO:0006163); Biological process: phosphorylation (GO:0016310) |
| c128056.graph_c0 | — | — | Nucleotide transport and metabolism |
| c113537.graph_c0 | — | — | — | DNA mismatch repair protein |
| c113537.graph_c0 | — | — | Transcription |
| c113537.graph_c0 | — | — | — | Myb transcription factor (MYB1) |
| c113537.graph_c0 | — | — | — | Putative_Transcription_factor |
| c110500.graph_c0 | — | — | — | Photosystem II (GO:0009523) |
| c50248.graph_c0 | — | — | — | Cellular component: vacuole (GO:0005773); Cellular component: Golgi apparatus (GO:0005775); Cellular component: integral component of membrane (GO:0016021); Cellular component: cytoplasmic membrane-bounded vesicle (GO:0016023) |
| c105628.graph_c0 | — | — | — | Myb_family_transcription_factor (MYB2) |
| c43510.graph_c0 | — | — | Secondary metabolites biosynthesis, transport, and catabolism |
| c133272.graph_c0 | — | — | — | Endoribonuclease Dicer homolog 1 |
| c111874.graph_c0 | — | — | — | GRAS family transcription factor |
| c102822.graph_c0 | — | — | — | Transcriptional_corepressor_SEUSS |
| c68622.graph_c0 | — | — | — | Hypothetical protein |
| c130130.graph_c0 | — | — | — | — |
| c194399.graph_c0 | — | — | Molecular function: binding (GO:0005488); Biological process: regulation of transcription, DNA-templated (GO:0006355) |
| c124197.graph_c0 | — | — | — | Hypothetical protein |
| c130130.graph_c0 | — | — | — | Helix-loop-helix DNA-binding domain |
| c130130.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | Chaperone protein dnaJ |
| c137022.graph_c0 | — | — | — | Putative uncharacterized protein |
| c111874.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | ATP-binding Glutathione S-conjugate transferase |
| c103359.graph_c0 | — | — | — | — |
| c149867.graph_c0 | — | — | — | — |
| c103359.graph_c0 | — | — | — | — |
| c130130.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | Uncharacterized protein |
| c111874.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
| c92818.graph_c0 | — | — | — | — |
| c133926.graph_c0 | — | — | — | — |
| c102363.graph_c0 | — | — | — | — |
| c107011.graph_c0 | — | — | — | — |
**Supplemental Table 5. Continued.**

| miRNA Accession no. | miRNA Family | Target accession no. | COG* class annotation | GO annotation | Nr annotation |
|---------------------|--------------|----------------------|------------------------|---------------|--------------|
| c120292.graph_c0    | miR7717      | c120292.graph_c0     | Chromatin structure and dynamics | —             | Hypothetical protein |
| c124819.graph_c0    | miR7492      | c124819.graph_c0     | RNA processing and modification | Molecular function: ribonuclease III activity (GO: 0004525); Biological process: RNA processing (GO: 0006396) | Ribonuclease 3-like protein 2 |
| c134394.graph_c0    | novel_miR_01 | c134394.graph_c0     | —                      | Molecular function: nucleotide binding (GO: 000166); Biological cellular component: vacuole (GO: 0005773); Cellular component: chloroplast (GO: 0000579); Molecular function: isomerase activity (GO: 0016553) | Hypothetical protein |
| c139609.graph_c0    | novel_miR_02 | c139609.graph_c0     | Coenzyme transport and metabolism | Molecular function: calcium-transporting ATPase activity (GO: 000538); Molecular function: ATP binding (GO: 0005524); Cellular component: integral component of membrane (GO: 0010021); Molecular function: metal ion binding (GO: 0046872) | ATPase family protein |
| c133751.graph_c1    | novel_miR_03 | c123122.graph_c0     | Carbohydrate transport and metabolism | —             | —             |
| c178187.graph_c0    | —            | c126858.graph_c0     | —                      | Molecular function: calcium ion transmembrane transport (GO: 0005058) | —             |
| c13112.graph_c0     | novel_miR_04 | c129764.graph_c0     | —                      | Molecular function: arginine transmembrane transporter activity (GO: 0015181); Biological process: arginine transport (GO: 0015809); Cellular component: integral component of membrane (GO: 0016021) | —             |
| c129357.graph_c0    | —            | c58199.graph_c0      | —                      | Squamosa promoter-binding-like protein 3 isoform X1 | —             |
| c107606.graph_c0    | novel_miR_05 | c12983.graph_c0      | —                      | Hypothetical protein | —             |
| c20277.graph_c0     | —            | c15025.graph_c0      | —                      | Hypothetical protein | —             |
| c119993.graph_c0    | —            | c157363.graph_c0     | Signal transduction mechanisms | Biological process: regulation of transcription, DNA-templated (GO: 006355); Biological process: response to salt stress (GO: 000651) | —             |
| c129372.graph_c0    | novel_miR_06 | c129372.graph_c0     | —                      | —             | —             |
| c115261.graph_c1    | —            | c12619.graph_c0      | —                      | Myb_DNA-binding domain-containing protein (MYB4) | —             |
| c123462.graph_c0    | —            | c123969.graph_c0     | —                      | Hypothetical protein | —             |
| c115067.graph_c0    | novel_miR_08 | c123969.graph_c0     | —                      | Hypothetical protein | —             |
| c115067.graph_c0    | novel_miR_09 | c128575.graph_c0     | General function prediction only | —             | —             |
| c40173.graph_c0     | —            | c131547.graph_c0     | —                      | Myb_DNA-binding protein (MYB5) | —             |
| c127282.graph_c0    | —            | c127282.graph_c0     | Signal transduction mechanisms | Biological process: cellular process (GO: 0009987); Biological process: single-organism process (GO: 0044699) | Importin-5-like |
| c126232.graph_c0    | novel_miR_10 | c115067.graph_c0     | Posttranslational modification, protein turnover, chaperones | —             | —             |

*Continued next page*
| miRNA Accession no. | miRNA Family | Target Accession no. | GO Annotation | NR Annotation |
|---------------------|--------------|---------------------|---------------|--------------|
| c82394.graph_c0_36819 | novel_miR_11 | c125877.graph_c0_36819 | Chromatin structure and dynamics | Lysine-specific demethylase 5B isoform X4 |
| c114547.graph_c0_6259 | novel_miR_17 | c149822.graph_c0_6259 | Replication, recombination and repair | Hypothetical protein |
| c120584.graph_c1_6259 | — | c130705.graph_c0_6259 | — | Hypothetical protein |
| c116141.graph_c0_6259 | — | c205686.graph_c0_6259 | Replication, recombination and repair | Hypothetical protein |
| c50327.graph_c0_32983 | novel_miR_14 | c109500.graph_c0_32983 | — | Delta-6 elongase |
| c115909.graph_c0_6259 | novel_miR_19 | c36563.graph_c0_6259 | General function prediction only | Zinc-finger protein, putative |
| c100429.graph_c0_140 | novel_miR_18 | c17547.graph_c0_140 | General function prediction only | F-box only protein 8 isoform X1 |
| c111239.graph_c0_3989 | novel_miR_19 | c192375.graph_c0_3989 | Defense mechanisms | ATPase family protein |
| c107685.graph_c0_140 | — | c23686.graph_c0_140 | — | Hypothetical protein |
| c113180.graph_c0_4822 | novel_miR_24 | c13599.graph_c0_4822 | Cellular component: plasma membrane | Ribulose bisphosphate carboxylase small chain, chloroplastic-like |

The mRNA accession no. could be obtained from small RNA sequences of *P. rockii* (PR) and *P. ostii* (PO) with NCBI accession number SRP102723. The target accession no. could be obtained from the transcriptome sequences of *P. rockii* and *P. ostii* with NCBI accession no. SRP106947. NCBI represents gene ontology.