Identification and characterization of microRNAs in tree peony during chilling induced dormancy release by high-throughput sequencing

Yuxi Zhang, Yanyan Wang, Xuekai Gao, Chunying Liu & Shupeng Gai

Tree peony, one of the most valuable horticultural and medicinal plants in the world, has to go through winter to break dormancy. Growing studies from molecular aspects on dormancy release process have been reported, but inadequate study has been done on miRNA-guided regulation in tree peony. In this study, high-throughput sequencing was employed to identify and characterize miRNAs in three libraries (6 d, 18 d and 24 d chilling treatments). There were 7,122, 10,076 and 9,097 unique miRNA sequences belonging to 52, 87 and 68 miRNA families, respectively. A total of 32 conserved miRNAs and 17 putative novel miRNAs were identified during dormancy release. There were 771 unigenes as potential targets of 62 miRNA families. Total 112 known miRNAs were differentially expressed, of which 55 miRNAs were shared among three libraries and 28 miRNAs were only found in 18 d chilling duration library. The expression patterns of 15 conserved miRNAs were validated and classified into four types by RT-qPCR. Combining with our microarray data under same treatments, five miRNAs (miR156k, miR159a, miR167a, miR169a and miR172a) were inversely correlated to those of their target genes. Our results would provide new molecular basis about dormancy release in tree peony.

Tree peony (Paeonia suffruticosa Andrews) is one of the earliest and most well-known horticultural and medicinal plants in the world. Since flower buds of tree peony must go through a period of bud dormancy before bud sprouting in spring, the common adopted measure in agriculture is breaking dormancy by artificial chilling treatment for forcing culture. According to Lang and Martin1, the dormancy in tree peony belongs to endo-dormancy similar to some temperate fruit plants like grape, apple, peach, kiwifruit and so on. A sufficient chilling duration during winter is the main mean to break dormancy and induce growth in the following spring by appropriate warmer temperature2.

Release of dormancy physiological status was controlled through cooperation of large number of genes in woody plants3. Liu et al. obtained 190 significantly differentially expressed genes associated with bud dormancy in pear4. In Chinese cherry, totally 1,644 significantly differentially expressed genes were identified based on RNA-seq transcriptome5. To discover transcriptional pathways associated with dormancy release in Prunus persica, Fu et al. explored the chilling-dependent expressions of 11 genes involved in endoplasmic reticulum stress and the unfolded protein response signal pathways6. Yordanov et al. suggested that EARLY BUD-BREAK 1 (EBB1) have a major and integrative role in reactivation of meristem activity after winter dormancy in poplar trees7. In tea, sequence and gene ontology analysis of 3,500 clones associated with dormancy were analyzed8. In recent years, growing studies from molecular aspects on tree peony endo-dormancy release process have been reported, such as Huang et al. screened 31 unigenes associated with dormancy release in tree peony by SSH (suppression subtractive hybridization)9. Gai et al. obtained over 15,000 high quality unigenes by RNA sequencing during chilling requirement fulfillment through Roche 454 GS FLX platform10, of which 3,174 genes were significantly differentially expressed during endo-dormancy release in tree peony11. More recently, Zhang et al. obtained 20 differentially expressed protein spots (P < 0.05) during dormancy release by combination of two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionisation time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS)12. In addition, Zhang et al. found a MADS-box member (SUPPRESSOR OF
OVEREXPRESSION OF CONSTANS, *PsSOC1*) that not only accelerates flowering, but also promotes dormancy release in tree peony13. However, endo-dormancy mechanisms in tree peony are still unclear despite many efforts.

Eukaryotic gene expression is mainly regulated at the transcriptional and post-transcriptional levels. MicroRNAs (miRNAs) are a specific class of small non-coding RNA of commonly 19–24 nucleotides involved in post-transcriptional gene expression regulation14. The mature miRNAs negatively regulate gene expression through complementary binding to the opening reading frame (ORF) or UTR regions of specific target genes. In plants, miRNAs generally interact with their targets through near-perfect complementarily, which leads to gene silencing by endonucleolytic cleavage or translational inhibition15–17. Recent studies indicate that miRNAs play important roles in plant developments including organ separation, leaf development and polarity, lateral root formation, floral organ identity and reproduction, etc.18–21. Zhang et al.22 identified differentially expressed miRNAs responding to cold stress in tea. Jeyaraj et al.23 analyzed the expression pattern of tea miRNAs in active and dormant bud using stem-loop pulse RT-qPCR method. In poplar, ptr-miR169 was found to repress ptrHAP2 at the level of post-transcription during vegetative bud dormancy period24. In tree peony, some of conserved and novel miRNAs were identified under copper stress25. However, no miRNAs have been reported especially during chilling endo-dormancy release in tree peony.

In this study, we aimed to identify and characterize miRNAs by high-throughput sequencing technology in tree peony during the period of bud dormancy release after 6 d, 18 d and 24 d chilling requirement fulfilling, which included three physiological status, endo-dormancy, endo-dormancy release and eco-dormancy10. Our results increase the available information on miRNA-guided regulation mechanism and physiological changes during chilling induced dormancy release in tree peony.

**Results**

**Deep sequencing of Paeonia ostii sRNAs.** To investigate small RNA expression profiles in *Paeonia ostii* during physiological dormancy stages based on the results of morphologic observations11, three small RNA libraries of flower buds were constructed after 6 d, 18 d and 24 d chilling enduring. For each library, small RNAs were collected, pooled together and sequenced. A total of 19,762,599 reads with lengths of 16 bp to 30 bp were obtained from the three libraries, and average 3.8 million (range: 2.69–5.05 million) clean small RNA reads were acquired from each library after removing adapters and low-quality reads. The average number of unique reads per library was 2.686,857, ranging from 1.63 to 5.07 million (Table 1). Most obtained sRNA sequences were 21–24 nt in all of the three libraries, of which 24 nt long sRNAs were the most abundant, accounting for approximately 52.3% on average (Fig. 1).

Table 1. Statistics of small RNA sequence reads.

| Different treatments | 6 d   | 18 d   | 24 d   |
|----------------------|-------|--------|--------|
| Title                | Number| percent| Number| percent| Number| percent|
| Total Tags number    | 5,338,004 | 100%    | 8,092,580 | 100%    | 6,332,015 | 100%    |
| Average quality < 13 Tags | 1,469,084 | 27.52%  | 1,577,909 | 19.50%  | 1,224,509 | 19.34%  |
| Length < 16          | 74,930 | 1.4%   | 204,694 | 2.53%   | 212,955  | 3.60%   |
| Length > 30          | 615,543 | 11.53%  | 427,666 | 5.28%   | 507,468  | 8.01%   |
| Clean number         | 2,686,857 | 50.33%  | 5,047,088 | 62.37%  | 3,770,180 | 59.54%  |
| Unique number        | 1,629,348 | 30.52%  | 2,683,551 | 33.16%  | 1,895,066 | 29.93%  |
and potential miRNA seeds (Table 2). It is noticeable that the miRNAs represented 19.62% of the total sRNA in 6 d chilling treatments, but only 14.01% and 14.78% in 18 d and 24 d chilling treatments, which may as a result of many genes associated with endo- and eco-dormancy release are activated during dormancy release. There are about more 9,000 unique miRNAs at the physiological stage of dormancy release and eco-dormancy than at the status of dormancy, which indicates that the miRNA populations in flower buds after dormancy release are more diversified, as well as biological processes are more complex.

### Nucleotide Preference of 19–24 nucleotide small RNAs.

Previous studies have shown that most miRNA sequences start with uridine (U), whereas the majority of 24-nucleotide siRNAs have adenosine (A) as their 5’ first nucleotide in plants. In our result, the same trends were observed among cloned tree peony small RNAs, about 67.1% of small RNAs sequences started with uridine, and all 24-nt siRNAs had start-nucleotide preference with adenosine (Fig. 2a). Besides, we found that about 45.5% of total small RNAs also had a clear preference for adenosine as the last nucleotide, of which all 23-nt had a clear preference for uridine and all 24-nt for adenosine as the last nucleotide (Fig. 2b). In order to investigate whether the last nucleotide preference from 19 nt to 24 nt small RNAs also exist in other model plants like Arabidopsis, we downloaded 427 Arabidopsis small RNA deep sequencing datasets from miRBase database (http://www.mirbase.org/ftp.shtml) and analyzed their nucleotide compositions (Supplementary Figure S1). In all 427 Arabidopsis datasets, strong last nucleotide preference for uridine was observed in 23 and 24 nt small RNAs, indicating that difference of nucleotide preference might exist among species.

### Identification of conserved and novel miRNAs in tree peony.

To identify conserved miRNAs in tree peony, but its genome is not available, known plant miRNAs (miRNA precursors and mature miRNAs) registered in miRBase 21.0 were used as reference for miRNA mapping. Clean data that aligned to known miRNAs allowing two mismatches and had no less than 5 reads per million (RPM) in at least one library were regarded as conserved miRNAs. In three libraries, total 112 known miRNAs belonging to 99 miRNA families were identified in the three libraries, of which there were 7,122, 10,076 and 9,097 unique miRNA sequences belonging to 52, 87 and 68 miRNA families, respectively (Additional file 1, Table 3). Of which, there was 32 conserved miRNAs (Table 4). In our data, 15 miRNAs sequences were found anchored in the 5p-arm and 17 miRNAs anchored in the 3p-arm (Addition file 1, Table 4). Unexpectedly, one less-conserved miRNA (miR5072) was obtained,
which was previously found only in monocots. Furthermore, there were 55 miRNAs belonging to 46 miRNA families shared in the three libraries (Fig. 3), and the most abundant miRNA identified by sequence homology was Psmir159 with more than 500,000 actual sequencing reads, accounting for approximately 69% of the total conserved miRNA reads, following by PsmirR5266 with more than 100,000 actual sequencing reads, Psmir166, Psmir319, Psmir1509 with more than 10,000 actual sequencing reads, and mir398 showed the minimum amount (Table 4). At the same time, the frequencies of miRNAs read varied from 8 (PsmiR2111a-5p) to 709,087 (Psmir159a), which indicate that miRNAs displayed drastically different expression level in tree peony during dormancy release (Fig. 3). After normalization, more than half of the conserved miRNAs were less than 100 times. In addition, the relative abundance of certain members within same miRNA family varied widely (Table 4). For instance, the normalized number of Psmir167a was 255, but that for Psmir167b was only 12. Furthermore, the normalized reads of different members in three treatments were significant different, for example, the abundances of members in mir159 (mir159a and mir159b–3p) ranged from 18,716, 180 (6 d), 20,170, 496 (18 d) to 22,752 and 218 (24 d) reads in three libraries, respectively (Table 4). These results suggest that members showed different expression trends within same miRNA family, probably because their expressions are development-stage specific or either induced or suppressed during dormancy release in tree peony.

Based on the miRNA annotation criteria, novel miRNAs could be predicted by mapping the remaining non-annotated sRNAs to Populus genome. In our data, seventeen novel miRNAs were obtained and named as Psmir1 to Psmir17 (Table 5). All precursors of novel miRNAs had regular stem-loop structures and the predicted hairpin structures. To investigate the conservation of these 17 novel miRNAs in other plant species including Malus domestica, Physcomitrella patens and Populus trichocarpa, they were used to perform BLAST searches against miRBase databases. Psmir5, Psmir7 and Psmir16 matched genomes of other plant species (Table 5). Reverse transcript PCR (RT-PCR) was performed to validate the expression of some new predicted miRNAs in flower buds after chilling treatments. The primer sequences were listed in Supplementary Table S1. We found five of the 17 predicted miRNAs including Psmir9, Psmir3, Psmir1, Psmir4 and Psmir13 expressed in tree peony flower buds (Fig. 4). PsU6 was amplified as a positive control. We found that these novel miRNAs could be detected in flower buds after 18 d chilling treatments.

We found 469 mature miRNAs could be aligned to other species’ precursors (precursors data from miRbase r21), mostly in Glycine max (36.7%), Oryza sativa (30.7%), and Arabidopsis thaliana (30.06%). The 17 precursors of novel miRNAs derived from tree peony transcriptome was listed in Table 5, only 1 precursors coded both 5p and 3p side mature miRNAs, others only possessed 1-side mature sequence.

Table 3. Summary of small RNA deep sequencing data. aLengths between 16–30 nt.

| Libraries | No. of sequences generated* | No. of non-redundant sequences* | No. of sequences with perfect matches to the miRBase | Unique miRNA number | Family number |
|-----------|-----------------------------|---------------------------------|-----------------------------------------------------|---------------------|--------------|
| 6 d       | 2,686,857                   | 1,629,348                       | 193,210                                             | 7,122               | 52           |
| 18 d      | 5,047,088                   | 2,683,551                       | 232,971                                             | 10,076              | 87           |
| 24 d      | 3,770,180                   | 1,895,066                       | 231,665                                             | 9,097               | 68           |

Prediction of miRNA targets in tree peony. Previous study found that plant miRNA target sites mainly situate at opening reading frames (ORFs). To understand possible biological functions of the identified miRNAs in tree peony, we predicted the miRNA targets using the mRNA transcriptome of Paeonia ostii flower buds as a reference sequence since the genome of Paeonia ostii is not publicly available. A total of 771 unigenes were predicted as potential targets of 62 known miRNA families (Additional file 2), and the majority of target proteins and corresponding annotations were shown in Table 6. Most miRNAs had more than one predicted target proteins, and some of the miRNAs have more than 10. Based on GO annotation, more than half of the predicted target genes were involved in biological process (metabolic process, regulation of transcription, signal transduction, transport and regulation of act polymerization) and molecular function (binding and methyltransferase activity) (Fig. 5). However, there were many conserved miRNAs target genes that had no functional annotation. Novel miRNAs targets were also predicted, but only two of them have been found target relationship with two unigenes (Table 5).

Expression profile of Paeonia ostii miRNAs during dormancy release. To identify miRNAs that were responsive during dormancy release, we compared miRNA expression level among three libraries. All the conserved candidate miRNAs with no less than 10 reads in each library were analyzed. Differentially expressed miRNAs that exhibited more than a 2-fold change were selected between each two treatments. There were 112 known miRNAs differentially expressed among three libraries (Additional file 3), and of which 55 miRNAs were shared among three libraries (See Additional file 3-shared miRNAs in three libraries). There existed 28 miRNAs (including mirR126-3p, mirR6479, mirR2949b, mirR5057, mirR6144, mirR7743-3p, mirR6483, mirR5083, etc.) only in 18 d chilling duration library, which might play important roles in regulation dormancy release.
| Family | miRNA IDs | sequences | Actual sequencing reads | zma | ath | osa | vvi | ptc |
|--------|-----------|-----------|------------------------|-----|-----|-----|-----|-----|
|        |           |           | 6d | 18d | 24d | zma | ath | osa | vvi | ptc |

**miR156**

- PsmiR156a
  - UGACGGAGAGAAGAGCAGCAC
  - 263/23
  - 51/4
  - 161/14

- PsmiR156b-3p
  - GCUCACUCUCUAUCUCUCAC
  - 0/0
  - 23/2

**miR159a**

- UUUGGAAUUGAAGGGACAGCUCA
  - 215,316/18,716
  - 232,033/20,170
  - 261,738/22,752

- AUUGGGAUGAAGGGGCUCCU
  - 2,067/180
  - 5,704/496
  - 2,505/218

**miR160a**

- UGGCCGCGUCUCGCUGAUAGCCA
  - 216/19
  - 1,071/93
  - 577/50

**miR160a-3p**

- CGCGUUGGAGGAGCAAGCUA
  - 60/5
  - 45/4

**miR162a**

- UUGAAUACGUUCUGAUACUCA
  - 551/48
  - 1,236/107

**miR162b**

- UUGAAUACGCGUCUGAUACUCC
  - 489/43
  - 954/83

**miR163b**

- UGGCCGCGUCUCGUGCAUGCCA
  - 112/10
  - 4/0

**miR164a**

- UGGAAGGGGAGGACGUGCA
  - 332/29
  - 409/36

**miR164b**

- UGGAAGGGGAGGACGUGCA
  - 2/0
  - 10/1

**miR164c**

- UGGAAGGGGAGGACGUGCA
  - 524/46
  - 8/1

**miR166a**

- CGCGAGCCCCGUCUCUCUCC
  - 3,665/319
  - 19,051/1,656

**miR167a**

- UGAGAUGCCAGACGUGAUGUCA
  - 714/63
  - 1,339/116

**miR167b-3p**

- GUGCAUGCGACGUGAUGUCA
  - 41/4
  - 56/5

**miR168a**

- UUGUGGAGGACGUGAUGGAGA
  - 738/64
  - 2,375/206

**miR169a-3p**

- CCCGGCUGUAACUCACUAAGAU
  - 151/13
  - 140/12

**miR169-3p**

- GCAUGUUGGUGCUUGCCUACA
  - 218/19
  - 837/73

**miR171a**

- UUGAUAGCCGCGGGACGUGAU
  - 277/24
  - 234/20

**miR171b-3p**

- UUGAGGCGGUGCUGGAUCAU
  - 141/12
  - 136/12

**miR172a**

- AGAUCUUAGAAGGACGUGCAU
  - 51/4
  - 96/8

**miR172a-3p**

- GUGCCGCGUCUCACAGACUCA
  - 2/0
  - 6/1

**miR319a**

- UGUGAUGCCAGACGUGAUGUCA
  - 0/0
  - 4,519/393

**miR319a-3p**

- UGUGAUGCCAGACGUGAUGUCA
  - 4,587/403
  - 11,200/974

**miR319b**

- UGUGAUGCCAGACGUGAUGUCA
  - 1,408/122
  - 0/0

**miR390a-3p**

- CGGCUUGACUCCUGACUCA
  - 26/2
  - 139/12

**miR390b-5p**

- AACUGCCGAGGCUAGGAACCC
  - 1,062/92
  - 2,193/191

**miR394**

- UUGCGCAGGACGACGGAA
  - 307/27
  - 1,266/110

**miR396-5p**

- UUCACUAGAAAGGUGGGAAA
  - 0/0
  - 27/1

**miR396c**

- UUCAAGAAAGUUCGGGAGGA
  - 48/4
  - 48/4

**Less-conserved**

- CGGGCTGGACUGCCGUGCGGC
  - 46,659/4,056
  - 39,563/3,439

- GCUGCCGUGGCUUCAUCUA
  - 0/0
  - 23/2

- UUGUUAUUGUAAUUGGGAGGAA
  - 70/6
  - 0/0

- UUUGAUCUUGGCGACAAUCCCA
  - 1,629/142
  - 3,659/318

- CCCCGACGGUGGGGCCGA
  - 22/2
  - 99/9

- UCCGUAGCAGCGACCA
  - 38/3
  - 200/17

- UCCGACUGGAGGCUCCAC
  - 288/25
  - 1,483/129

- AACCUGCCGUGUCAUCA
  - 113/10
  - 425/37

- UCCUGCCGUGUCAUCA
  - 293/25
  - 1,228/107

- UUGGAAUUGUAAUGUGGAGA
  - 48/4
  - 205/18

- AUUGAUGACUGAAAGGACC
  - 1,898/94
  - 640/56

- AAUCUGUGAAGAGGACC
  - 51/4
  - 103/9

- UGAAACCUAGAAAGAGCUGG
  - 2,367/206
  - 4,552/396

- UUAGGAUAAUGUCCAGAACC
  - 28/2
  - 44/4

- AUUGGGAUAGGAGACGAC
  - 954/83
  - 2,398/208

- AAUGAGAUGAUGAGACAC
  - 1,738/151
  - 1,553/135

- CCGACCCUGUGAGGUGUAGA
  - 64/6
  - 270/23

- UUUCAAGUUAUUGCCAGAACC
  - 942/43
  - 778/68

- UUGUGUUGUCUGCUGCUGAGG
  - 41/4
  - 80/7

- UUGUGUUGUCUGCUGACCAA
  - 131/11
  - 520/28

**Continued**
Table 4. Known miRNAs identified from tree peony flower bud after different chilling treatments. 0 represents no mismatch, + represents one mismatch, ++ represents two mismatches, and so on. zma, Zea mays; ath, Arabidopsis thaliana; osa, Oryza sativa; vvi, Vitis vinifera; ptc, Populus trichocarpa.

| Family ID | miRNA IDs | sequences              | Actual sequencing reads | Normalized sequencing reads | zma | ath | osa | vvi | ptc |
|-----------|-----------|------------------------|-------------------------|-----------------------------|-----|-----|-----|-----|-----|
| miR6300  | PsmiR6300 | GUCCGUUGUAACAGUGG      | 10,410/905              | 0/0                         | NA  | NA  | NA  | NA  | NA  |
| miR398   | PsmiR398b-3p | UUGUGUCUCAGGGACCCCU  | 13/1                    | 21/2                        | 39/3| NA  | 0   | NA  | 0   |
| miR5072  | PsmiR5072 | AACGACUCAGAGUGUCC      | 23/2                    | 178/15                      | 622/54| NA  | 0   | NA  | 0   |

Figure 3. Abundance of most conserved miRNA families in three libraries from tree peony buds after 6 d, 18 d and 24 d chilling treatments.

Expression Validation of tree peony miRNAs. To confirm the expression patterns of miRNAs, as well as detect their responses to chilling treatments at three physiological stages, the expression of 15 conserved miRNAs, whose sequencing counts altered significantly after treatment, were analyzed by RT-qPCR. It is showed that the expression levels of miRNAs were a constant change process with the time of treatment. We classified them as detect their responses to chilling treatments at three physiological stages, the expression of 15 conserved miRNAs, whose sequencing counts altered significantly after treatment, were analyzed by RT-qPCR. It is showed that the expression levels of miRNAs were a constant change process with the time of treatment. We classified them into four types (Fig. 7). Type a: slowly increased (PsmiR3630, PsmiR398b-3p, PsmiR159a and PsmiR166a); type b: suddenly increased (PsmiR168a and PsmiR5072); type c: first increased and then decreased (PsmiR159b-3p, PsmiR160a, PsmiR166a, PsmiR167a, PsmiR169a, PsmiR319-3p and PsmiR172a); type d: first decreased and then increased (PsmiR156k and PsmiR157a). These results suggest that miRNAs belonging to type c were early stage...
Table 5. Candidate novel miRNAs in tree peony. ppt: Physcomitrella patens; ptc: Populus trichocarpa; mdm: Malus domestica.

| novel miRNA | mature sequence | other species' ID in miRbase | MFE (kcal/mol) | MFEI | Predicted precursors | p-value | Predicted target ID | Normalized miRNA abundance
|-------------|----------------|-----------------------------|----------------|------|----------------------|---------|-------------------|-----------------------------|
| PsmiR1-5p   | AGGGACTCTTTCCTCCTCCTC   | —                           | −81.9          | 1    | JI448260:47:0.214:+  | 0.99    | —                 | 7,624.35                    |
| PsmiR2-5p   | CATACTTCGGATAAGCG      | —                           | −11.2          | 0.4  | JI455606:1198:1247:+ | 0       | JI455773          | 4.78, 2.65                  |
| PsmiR3-5p   | GGTGACTGCTGCAAGGCCC   | —                           | −27.8          | 0.9  | JI443786:93:136:+    | 0.99    | JI450527          | 23,008.13, 159,333.51       |
| PsmiR 4-3p  | TATGAGACCTTTGCCAGAGGCCAC   | —                           | −37.9          | 1    | JI451506:405:481:-   | 0.99    | JI445930          | 3,384.18, 2,348.2, 2,987.29 |
| PsmiR 5-5p  | AGAGAATTGAAGTAGACGCCTAC   | ppt-miR1023b-3p              | −41.2          | 0.7  | ContiG02457:19:227:+ | 0       | JI446191          | 0, 0, 2.65                   |
| PsmiR 6-3p  | ATCTCTTTAGCTGAAGAAGGACC   | —                           | −66.4          | 0.5  | JI458593:131:405:-   | 0       | JI458593          | 2.65                        |
| PsmiR 7-3p  | AAGCCATGAGAAGCTAT        | ptc-miR169y                 | −82            | 0.7  | JI448255:1023:1305:- | 0       | JI450838          | 4.78, 0                     |
| PsmiR 8-5p  | TGTACTCAAGGTTAGAAAGAAGA   | —                           | −24.3          | 0.9  | JI456175:234:311:+   | 0.99    | JI452932          | 79.63, 45.43, 108.48       |
| PsmiR 9-5p  | CGTGGACTGCTGCAAGGCCC     | —                           | −28.9          | 0.9  | JI443786:93:137:-    | 0.99    | JI443786          | 374,494.16, 410,756.3, 374,225.73 |
| PsmiR 10-5p | AGCCTCTTGGTTGCCAGGCC   | —                           | −73.2          | 1.3  | JI448950:74:188:-    | 0       | JI448950          | 23.89, 253.47, 58.21        |
| PsmiR 11-5p | AGCTTTTGTATGTTCCTCGTTA  | —                           | −57.7          | 0.6  | JI452338:1477:1738:+ | 0       | 2.39             | 0                           |
| PsmiR 12-5p | GGTGAGATGATGACGCACCT    | —                           | −47.6          | 0.6  | JI454613:413:566:+   | 0       | JI453131          | 2.39, 0                     |
| PsmiR 13-5p | TTTGTGGTACCTCTTCAGACGA  | —                           | −63.9          | 1.5  | JI444316:158:242:+   | 0       | JI444316          | 1,282.01, 4,421.41, 828.18 |
| PsmiR 14-3p | CGACTGGGGAATTTGATTGGGGA  | —                           | −80            | 0.6  | JI454686:1279:1546:- | 0       | JI454686          | 0, 2.65                     |
| PsmiR 15-5p | AGGGCCATGCTCACTGGGCTCT  | —                           | −49.4          | 0.5  | JI458732:712:921:-   | 0       | JI458732          | 2.39, 0                     |
| PsmiR 16-3p | AGAAGAGAAAGAGAGAGAAGAGA | mdm-miR169a                 | −29.9          | 0.6  | JI452600:5:140:-     | 0       | JI457551          | 0, 2.65                     |
| PsmiR 17-3p | CCAAGTTAAAGCTCGCGAGAGAGA | —                           | −10            | 0.4  | JI450973:261:299:+   | 0       | JI453625          | 2.39, 0                     |
| PsmiR 13-3p | TGTGCCAGATTCACAAAACAAA | —                           | −63.9          | 1.5  | JI444316:158:242:+   | 0       | JI444316          | 557.39, 2,529.94, 494.79    |

Table 4. Predicted miRNA precursors and other parameters.

Figure 4. Reverse transcript PCR (RT-PCR) electrophoresis results for expression identification of novel miRNAs in flower buds after 18 d chilling treatments. In total, 5 of 17 novel miRNAs were confirmed by Reverse transcript PCR (RT-PCR) with 40 cycle-amplification. The sizes of PCR products were about 100 bp. PsU6 was used as positive control.

Discussion

Tree peony is an important horticultural crop worldwide of great ornamental, medical and economic value. Native to China, tree peony is regarded as the “King of flower” and have deep botanical history in Chinese culture. It is crucial to understand the molecular mechanism of dormancy, which is a main obstacle for tree peony forcing culture. Based on morphological changes of Paeonia ostii ‘Feng Dan’ and global mRNA expression profiling, the physiological status of flower buds receiving less than 18 d chilling treatment are regarded as endo-dormancy, and that receiving more than 18 d are defined as eco-dormancy. miRNAs are paid more and more attention as key regulators of gene activity in animals and plants. In this study, we adapt high-throughput sequencing technology to identify sRNAs from Paeonia ostii and analyze their response to dormancy release. This work will provide useful information to deepen our understanding of the miRNA regulatory mechanisms during dormancy release.

There are 243, 511 and 207 miRNAs annotated in Arabidopsis, rice and soybean according to the miRBase database, respectively. In this study, we first completed construction of sRNA libraries (6 d, 18 d and 24 d chilling treatments) in tree peony and obtained over 19 million 16–30 nt reads. The size distribution of sRNAs revealed that 21, 22, 23 and 24 nt sRNAs were relatively abundant, of which 24 nt sRNAs were significantly higher...
than others. Similar results were observed from most plants, such as Arabidopsis, rice, tomato, cucumber, apple, peach, and rose. However, 21 nt-long sRNAs were the second enriched in this study, which was different with previous reports in Arabidopsis (Arabidopsis thaliana) and rice (Oryza sativa). But in poplar, the 21 nt sRNAs are the most abundant. Most of 21 nt sRNAs in our data started with uridine, which was consistent with the observation that ARGONAUTE1 (AGO1) usually harbors miRNAs with a 5′ terminal uridine. 24 nt sRNAs had start-nucleotide preference of adenosine, which was also reported in previous work. Tree peony endo-dormancy transcriptome database was employed to predict putative targets of tree peony miRNAs. The well-known conserved miRNAs including miR156, miR159, and miR164 have been identified. 

### Table 6. Majority of the predicted target genes and corresponding annotation of known miRNAs in tree peony.

| miRNA family | Target ID | Targets annotation | miRNA family | Target ID | Targets annotation |
|--------------|-----------|--------------------|--------------|-----------|--------------------|
| miR1509      | JI451099  | Peroxiredoxin      | miR319       | JI449827  | AP2 domain-containing transcription factor |
| miR156       | JI447102  | DNMT2 (DNA METHYLTRANSFERASE-2) | miR319 | JI447690 | Polygalacturonase precursor |
| miR156       | JI446831  | SQUAMOSA promoter-binding protein-like | miR395 | JI453154 | beta galactosidase |
| miR159       | JI446967  | RAB6A; GTP binding/protein binding | miR396 | JI445772 | Chitin-inducible gibberellin-responsive protein |
| miR159       | JI446401  | asparagine synthetase | miR396 | JI444318 | glyceraldehyde 3-phosphate dehydrogenase |
| miR162       | JI449996  | ubiquitin           | miR397       | JI455403 | lipoxigenase |
| miR164       | JI458131  | PID2 (PINOID2); ATP binding/protein kinase | miR414 | JI453820 | zinc finger protein |
| miR164       | JI449546  | dtp-glucose 4-6-dehydratase | miR414 | JI445308 | phosphoesterase |
| miR166       | JI446960  | pentatricopeptide repeat-containing protein | miR414 | JI444902 | Phospho-2-dehydro-3-deoxyheptonate aldolase 1 |
| miR167       | JI458177  | transmembrane protein | miR5059 | JI445796 | CAM7 (CALMODULIN 7); calcium ion binding |
| miR167       | JI452318  | tryptophan synthase alpha subunit | miR5083 | JI446932 | COP1-interacting protein-related |
| miR167       | JI451976  | Serine/threonine-protein kinase PBS1 | miR5658 | JI455720 | Serine/threonine-protein kinase SAPK10 |
| miR168       | JI451707  | GRAS family transcription factor | miR5658 | JI454156 | dolichyl glycosyltransferase |
| miR169       | JI454583  | similar to Protein kinase | miR5658 | JI451464 | Stromal cell-derived factor 2 precursor |
| miR169       | JI450321  | Acetyl glucosaminyl transferase | miR6113 | JI450180 | Lactoyl glutathione lyase |
| miR169       | JI447773  | F-box family protein | miR6300 | JI450877 | 3-dehydroquinate synthase |
| miR169       | JI445119  | calcium-dependent protein kinase | miR6300 | JI450707 | DNA damage checkpoint protein |
| miR171       | JI449485  | endoglucanase | miR6300 | JI448956 | ankyrin repeat domain protein |
| miR172       | JI445772  | Transcription factor GRAS | miR8175 | JI446423 | aminobutyrate aminotransferase |
| miR172       | JI446524  | AP2 domain-containing transcription factor | miR845 | JI448900 | cell division protein |

Figure 5. Go analysis of targets of known miRNAs in this study.
However, nearly half of known miRNAs and three novel miRNAs were not predicted homologous to any proteins in the Genbank nr database, which might because of the incomplete tree peony genome and limited number of transcript data in public database. Hundreds of miRNAs have been surveyed since high-throughput sequencing technology is widely used, but little has been done on identifying and analyzing miRNAs in tree peony and their response during dormancy release. Total 112 known miRNAs belonging to 99 families were identified in the three libraries. Two miRNAs families, PsmiR159 and PsmiR166, were relatively abundant. PsmiR166a increased continuously until dormancy release (6 d–18 d) and had a very low expression level at eco-dormant stage (24 d), which was consistent with recent studies in poplar34,50. For the expression level of 17 novel miRNAs, PsmiR1 was continuously up-regulated from dormancy to eco-dormancy stage, PsmiR9, PsmiR10 and PsmiR13 (up-regulation) and PsmiR3 and PsmiR4 (down-regulation) had converse expression pattern at the early stage of dormancy release.

**Cold-responsive and auxin-related miRNAs.** A continuous chilling accumulation is an effective natural or artificial way to release dormancy in tree peony. The endo-dormant bud can respond to chilling treatment, which stimulates growth-promoting respond signals including auxin or appropriate outside conditions (such as exogenous GA). In our study, there were 112 conserved miRNAs differentially expressed between 6 d and 18 d chilling library and between 18 d and 24 d chilling library. Among them, PsmiR160a was highly expressed in endo-dormancy release (18 d chilling) and quickly decreased in eco-dormancy (24 d chilling). MiR160 targeted auxin response factor 10/16/17 (ARF10/ARF16/ARF17) and negatively regulated auxin signaling53,54. Ding et al.

| miRNA        | Dormancy release | Endo-dormancy | miRNA        | Dormancy release | Endo-dormancy | miRNA         | Dormancy release | Endo-dormancy |
|--------------|------------------|---------------|--------------|------------------|---------------|--------------|------------------|---------------|
| PsmiR5072    | 7.73913          | 3.494382      | PsmiR159b-3p | 2.75955          | −2.27705      | PsmiR167b-3p  | 3.16854         | −1.69697      |
| PsmiR390a-3p | 5.346154         | −1.20206      | PsmiR6284    | 2.513627         | −1.59336      | PsmiR1509b     | 1.239167        | −35.2232      |
| PsmiR559     | 5.263158         | 1.03           | PsmiR894     | 2.442748         | 1.65625       | PsmiR166a      | 1.231928        | 2.200489      |
| PsmiR170-5p  | 5.263158         | −1.83486      | PsmiR319a-3p | 2.441683         | −1.507        | PsmiR3627-5p  | 1.084906        | −1.29213      |
| PsmiR166a    | 5.19809          | −2.40149      | PsmiR403     | 2.246163         | −1.37453      | PsmiR159a      | 1.077639        | 1.128021      |
| PsmiR2109    | 5.153846         | −2.88795      | PsmiR2637    | 2.245283         | −2.83333      | PsmiR359c      | 1               | 1.520833      |
| PsmiR577     | 5.149306         | 1.031018      | PsmiR162-3p  | 2.243194         | −1.35378      | PsmiR171b-3p   | −1.03676        | 1.316476      |
| PsmiR160a    | 4.958333         | −1.85615      | PsmiR300b-5p | 2.064972         | 1.580483      | PsmiR168a-3p   | −1.07857        | 2.107143      |
| PsmiR166b-3p | 4.76017          | −1.26356      | PsmiR6108c   | 2.019608         | −2.28889      | PsmiR6441      | −1.11912        | 2.524147      |
| PsmiR165a    | 4.607143         | −2.93182      | PsmiR858b    | 1.95122          | −2.66676      | PsmiR5266      | −1.17936        | 1.447362      |
| PsmiR5054    | 4.5              | 1.636346      | PsmiR162a    | 1.95092          | −1.58472      | PsmiR171a      | −1.8376        | 1.478632      |
| PsmiR5371-5p | 4.270833         | −7.95259      | PsmiR6113    | 1.923109         | −1.81789      | PsmiR160a-3p   | −1.33333        | 1.088889      |
| PsmiR6478    | 4.21875          | −1.44385      | PsmiR172a    | 1.882353         | −1.14286      | PsmiR5558      | −1.69531        | 1.129688      |
| PsmiR5213-5p | 4.191126         | −2.0264       | PsmiR167a    | 1.87535          | −1.51471      | PsmiR1881      | −1.95918        | 2             |
| PsmiR394a    | 4.123779         | −3.28831      | PsmiR2105    | 1.797872         | −1.67327      | PsmiR2916      | −2.56452        | 3.516129      |
| PsmiR169b-3p | 3.83945          | −1.36098      | PsmiR398b-3p | 1.615385         | 1.857143      | PsmiR157a      | −3.38182        | 1.963636      |
| PsmiR5139    | 3.761062         | −3.57143      | PsmiR3630-3p | 1.585366         | 1.307692      | PsmiR356k      | −5.15686        | 3.156863      |
| PsmiR169a    | 3.4              | −2.20779      | PsmiR7984a   | 1.581301         | 1.224936      | PsmiR5519      | 2.25            | 0             |
| PsmiR168a    | 3.218157         | 1.351579      | PsmiR6279    | 1.571429         | −0.29412      |               |                 |               |

Table 7. Differentiated expressions of shared miRNAs from tree peony flower bud after different chilling treatments. Note: + and − indicate the induction and repression of miRNA, respectively.
found that \textit{miR160} was highly expressed in eco-dormancy (five weeks cold treatment)\textsuperscript{34}. In our case, the high expression of P\textit{smiR160} in endo-dormancy release might because of the difference of dormancy mechanism exist in tree peony and poplar. On the other hand, the expression of \textit{miR160} in endo-dormancy release may strengthen the auxin signal by inhibition of its target genes, and this hypothesis is also consistent with earlier report that

Figure 7. Validation of miRNAs expression patterns by Reverse transcriptase quantitative PCR (RT-qPCR) and expression patterns of partially corresponding target genes in our microarray results at three physiological stages. Type (a) slowly increased; type (b) suddenly increased; type (c) first increased and then decreased; type (d) first decreased and then increased. (e) Expression patterns of partially corresponding target genes in our microarray results.
exogenous GA could effectively promote endo-dormancy release\textsuperscript{19}. Ding et al. reported other two auxin-related miRNAs, miR390 and miR167, increased during active growth\textsuperscript{20}. In our study, the expression of PsmiR390b was steadily increased during the transition from endo-dormancy to eco-dormancy, and PsmiR167a was significantly induced during dormancy release, which suggest that auxin signal pathway participated in the process of dormancy release. We also found that PsmiR168a was continually up-regulated from endo-dormancy stage to eco-dormancy stage, the same trend was found in poplar\textsuperscript{34}. Similarly, miR168, member of the Csn-miR168 family, was found to be a cold-responsive miRNA, which was induced in two tea cultivars after 12 h of cold treatment\textsuperscript{22}. MiR168 regulates its target ARGONAUTE1 (AGO1) to participate in miRNA biogenesis\textsuperscript{37}. The high expression level of PsmiR168a would lead to the repression of AGO1, which would cause a reduction in the miRNA expression levels. The up-regulation of PsmiR168a suggested that cold-responsive miRNA participated in the release of endo-dormancy, their inductions were also consistent with its function of miRNA biogenesis\textsuperscript{37}.

**MiRNA targets.** Since the genome of *Paeonia ostii* is not publicly available, the miRNA transcriptome of *Paeonia ostii* flower buds\textsuperscript{11} were employed as a reference to predict the putative miRNA targets. Based on GO annotation, more than half of the predicted targets in tree peony were involved in binding, catalytic activity, metabolic process and cellular process. For example, miR5141 targets gene encoding ATP synthase, which have been reported to be involved in ATP synthesis and ATP utilization during seed dormancy breaking\textsuperscript{26}. In pear, specific enrichment of unigenes was observed for 15 pathways involved in metabolic processes including oxidative phosphorylation\textsuperscript{17}. Several other target transcripts, which encode alpha N-terminal protein methyltransferase, Endoglucanase, GTPase-activating protein and F-box domain associated with various biological processes and cellular activities were also detected. For instance, PsmiR395 targets genes encoding enzyme beta galactosidase, which recently have been reported to be involved in cell wall modification during the transition from dormancy to eco-dormancy in onion bulbs\textsuperscript{62}. PsmiR171 targets genes encoding endoglucanase, which had been shown to be antifreeze proteins during seed germination in sunflower\textsuperscript{55}. F-box proteins, the target of PsmiR169, have been identified previously as a key regulator of karrikin signaling and seed dormancy in Arabidopsis\textsuperscript{56}. Novel miRNAs target genes were also predicted, but only two of them have not been found target relationship.

MiR169 and miR166 regulated cellular process and biological process by acting on their target genes. MiR166 function mainly in vascular development\textsuperscript{60}, and the down-regulation of PsmiR166a at eco-dormant stage might help to increase the expression level of its target gene. In addition, Potkar et al.\textsuperscript{61} found that ptrmiR169a and its target gene PtrHAP2-5 showed inverse expression patterns during the dormancy period, which suggests that miR169 mediate attenuation of the target HAP2-5 transcript at this process\textsuperscript{24}. Jeyaraj et al.\textsuperscript{62} found that CsmiR169 targeted COBRA-like protein encoding gene and regulated cellulose synthase, which suggests that miR169 have possible role in cell cycle and other biological function during the bud development\textsuperscript{53}. In our study, PsmiR169a was highly expressed at the early stage of dormancy release and steadily down regulated at eco-dormant stage, and similar results were obtained during vegetative bud dormancy period of aspen\textsuperscript{54}.

MiR156 and miR172 regulate and control the juvenile-to adult vegetative transition by targeting transcription factors SQUAMOSA promoter-binding protein-like (SPL) and APETALA2 (AP2) genes in both annual herbs\textsuperscript{57,58} and woody perennial plants\textsuperscript{59}, showing converse expression patterns and regulatory relationships\textsuperscript{62}. It is noteworthy that in our study we found the expression levels of PsmiR156k and PsmiR172a during the transition from endo-dormancy to eco-dormancy also had the converse expression patterns. Similarly, Ding et al.\textsuperscript{60} also found miR156 and miR172 showed completely converse expression patterns during the dormancy-active growth transition\textsuperscript{34}. Our results showed that target genes (JH446524 and JH446831, putative AP2 and SPL genes) had cleavage sits of PsmiR172a and PsmiR156a, respectively, which suggested that miR156 and miR172 might play an important role during dormancy transition, which need to be further confirmed by experiments.

**Materials and Methods**

**Plant materials.** Four-year-old tree peony plants (*Paeonia ostii* ‘Feng Dan’) were potted and moved to refrigeration house with temperature 0–4 °C from 5 November to 30 December, 2014 in Qingdao, Shandong, China. The morphologic observation indicated flower buds receiving less than 18 d chilling treatment are in physiologic status of endo-dormancy, while those receiving more than 18 d chilling treatment were in eco-dormancy physiological status\textsuperscript{34}. Therefore, in order to decrease individuality, more than 5 plants were mixed buds-three generations house with temperature 0–4 °C from 5 November to 30 December, 2014 in Qingdao, Shandong, China.

**Small RNA library construction and sequencing.** Total RNA from tree peony flower buds after chilling treatments (6 d, 18 d and 24 d) was extracted using TRizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instruction and separated on 15% denaturing polyacrylamide gels. The 16–30 nt sRNAs were excised and recovered. The adapters (5’ and 3’) were ligated to the isolated sRNAs, which were sequentially reverse transcribed and amplified by PCR. The purified PCR products were sequenced using an Illumina Genome Analyzer (Illumina, USA) at Beijing Biomarker Technologies, Beijing, China.

**Analysis of sequencing data.** Raw sequence reads were produced by Illumina Genome Analyzer at Biomarker-Beijing, China and processed into clean full length reads by the Biomarker small RNA pipeline. During this procedure, all low-quality reads including 3’ adapter reads and 5’ adapter contaminations were removed. The remaining high-quality sequences were trimmed of their adapter sequences. Sequences larger than 30 nt and smaller than 16 nt were discarded. All high-quality sequences were considered as significant and further analyzed.

All matched sRNA sequences were categorized into classes including miRNAs, siRNAs, ribosomal RNAs (rRNAs), tRNAs, snRNAs, snoRNAs, repeat-associated sRNAs and degrade tags of extrons of introns, etc. Then,
the clean sequences were annotated by performing BLASTn searches against the Rfam (http://www.Sanger.ac.uk/Software/Rfam) and NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) databases, the detailed processes were following: the clean data were aligned with known miRNAs (miRNA precursors and mature miRNAs) registered in miRBase 21.0 (http://microrna.sanger.ac.uk/sequence/index.html) because of the difference among species, this process allowed two mismatches and free gaps to get temporary miRNA sequences and count of miRNA families. The highest-expression miRNA for each temporary mature miRNA family were selected to form a temporary miRNA database of *Paeonia ostii*. Finally, alignment of clean data to temporary miRNA database to identify conserved miRNAs in *Paeonia ostii*, only those perfect matching (≤ two mismatches) were considered as conserved miRNAs. Potential novel miRNAs were identified using criteria that were previously developed for plant miRNA prediction. The unique fold back structures of miRNA precursors can be utilized to predict novel miRNAs using MIREAP program (http://sourceforge.net/projects/mireap/). Potential targets for both known and novel miRNAs were identified on TAIR and Target Finder based on *Paeonia ostii* transcriptome sequencing data according to the search algorithm that only three or fewer mismatches and no gap are allowed to be present in the complementarily between miRNAs and their corresponding targets. The biological function category of the predicted targets was annotated against the Universal Protein Resource (http://www.uniprot.org).

**Differential expression analysis of miRNA and Reverse Transcriptase quantitative PCR (RT-qPCR) and 5′ RLM-RACE.** Differential expression analysis of miRNAs was performed based on sequence reads generated from three libraries after different chilling treatments according to the method described by Ren. In detail, the expression of miRNAs was normalized to obtain the number of miRNAs per million reads [normalized expression = (number of miRNA reads/total number of clean reads) × 1,000,000]. Normalized miRNA reads with values less than one in three libraries were excluded. The remaining miRNAs were used to calculate differences in expression by fold change (normalized miRNA reads in 18 d or 24 d chilling treatment/normalized miRNA reads in 6 d chilling treatment) and significant P-values. To validate miRNA expression, sRNAs were isolated from flower buds after different chilling treatments using an RNAiso for small RNA (TaKaRa, Dalian, China) following the manufacturer’s instructions. Then, the sRNA was polyadenylated by poly (A) polymerase, and first-strand cDNA was obtained using SYBR® Primesscript miRNA RT-PCR Kit (TaKaRa, Dalian, China). Briefly, the polyA was added to the 3′ of total RNA, then the RNA was reverse-transcribed with an oligo-dT adaptor. Quantitative PCR was performed in a total volume of 25 μL, containing 2 μL cDNA, 0.4 μM PCR forward primer (1 μL), 0.4 μM Uni-miR RT-qPCR primer (1 μL), 12.5 μL of 2 × SYBR premix Ex Taq II, and 8.5 μL dd H₂O. The reactions were completed using Roche Light Cycler 480 (Roche, Mannheim, Germany) with the following program: 95 °C for 10s and 40 cycles of 95 °C for 5s, 55 °C for 10s and 72 °C for 15s. The reactions were run in triplicate and the 2^−ΔΔCt relative quantification method was used to calculate the relative changes in gene expression. Small nuclear RNA U6 was used as endogenous reference, primers used in this study were listed in Supplementary Table S1.

To conform whether the predicted targets were cut by miRNAs and cleavage sites, the 5′ RLM-RACE were carried out using the FirstChoice RLM-RACE Kit (Ambion). Specifically, one microgram total RNA was firstly ligated to 5′ RACE oligo adaptor without calf intestine alkaline phosphatase and tobacco acid pyrophosphatase treatments. Then, the ligated RNA was used to synthesize the cDNA. The primers of miR172a target gene (JH46524) (5′-TCGAGAATGCTTGTCCATGGCAT-3′) and miR156a target gene (JH46831) (5′-TTGGAGGTTCGTGGTTGGAG-3′) for 5′ RLM-RACE were designed by Primer premier 5.0 software (Supplementary Table S1). PCR was carried out according to the manufacturer instructions, and the PCR products were purified by 1.0% agarose gel electrophoresis and cloned into the pMD18-T vector (Takara, Dalian, China) for sequencing.

**Availability of Data and Materials.** Our data have been presented in the main paper or additional supporting files.

**References**

1. Lang, G.A., Early, J.D., Martin, G.C. & Darnell, R.L. Endo-para-, and ecodormancy: physiological terminology and classification for dormancy research. *Hortscience.* 22, 371–377 (1987).

2. Rallo, L. & Martin, G.C. The role of chilling in releasing olive floral buds from dormancy. *Hortscience.* 26, 1058–1062 (1991).

3. Rohde, A. et al. *Molecular Aspects of Bud Dormancy in Trees, 89–134,* (Springer Netherlands, 2000).

4. Liu, G. et al. Transcriptomic analysis of ‘Suli’ pear (Pyrus pyrifolia white pear group) buds during the dormancy by RNA-Seq. *BMC Genomics.* 13, 700, https://doi.org/10.1186/1471-2164-13-700 (2012).

5. Zhu, Y. et al. RNA-Seq-based transcriptome analysis of dormant flower buds of Chinese cherry (*Prunus pseudocerasus*). *Gene.* 555, 362–376 (2015).

6. Fu, X. L. et al. Roles of endoplasmic reticulum stress and unfolded protein response associated genes in seed stratification and bud endodormancy during chilling accumulation in *Punica persica*. *Plos One.* 9, e101808, https://doi.org/10.1371/journal.pone.0101808 (2014).

7. Yordanov, Y.S., Ma, C., Strauss, S. H. & Busov, V. B. EARLY BUD-BREAK 1 (EBB1) is a regulator of release from seasonal dormancy in poplar trees. *Proc. Natl. Acad. Sci. USA.* 111, 10001–10006 (2014).

8. Thirugnanasambantham, K., Prabu, G., Palanisamy, S., Chandrabose, S. R. & Mandal, A. K. Analysis of dormant bud (Banjhi) specific transcriptome of tea (*Camellia sinensis* (L.) O. Kuntze) from cDNA library revealed dormancy-related genes. *Appl. Biochem. Biotech.* 169, 1405–1417 (2013).

9. Huang, X. et al. Genes associated with the release of dormant buds in tree peonies (*Paeonia suffruticosa*). *Acta Physiol Plant.* 30, 797–806 (2008).

10. Gai, S. et al. Transcriptome analysis of tree peony during chilling requirement fulfillment: Assembling, annotation and markers discovering. *Gene.* 497, 256–262 (2012).

11. Gai, S., Zhang, Y., Liu, C., Zhang, Y. & Zheng, G. Transcript profiling of *Paeonia ostii* during artificial chilling induced dormancy release identifies activation of GA pathway and carbohydrate metabolism. *Plos One.* 8, e55297, https://doi.org/10.1371/journal.pone.0055297 (2013).
51. Liu, P. U.

52. Mallory, A. C., Bartel, D. P. & Bartel, B. MicroRNA-directed regulation of Arabidopsis AUXIN RESPONSE FACTOR17 is essential.

50. Ko, J. H., Prassinos, C. & Han, K. H. Developmental and seasonal expression of PtaHB1, a gene encoding a class III HD-Zip gene, is associated with secondary growth and inversely correlated with the level of microRNA (miR166).

48. Zhao, Y. T.

47. Zhu, Q. H.

44. Martínez, G., Forment, J., Llave, C., Pallás, V. & Gómez, G. High-throughput sequencing, characterization and detection of new and conserved Cucumber miRNAs.

43. Zuo, J.

42. Zhang, B., Pan, X., Cannon, C. H., Cobb, G. P. & Anderson, T. A. Conservation and divergence of plant miRNA genes.

41. Griffiths-Jones, S., Saini, H. K., Dongen, S. V. & Enright, A. miRBase: tools for microRNA genomics.

40. Griffiths-Jones, S., Saini, H. K., Dongen, S. V. & Enright, A. miRBase: tools for microRNA genomics.

39. Vaucheret, H., Mallory, A. & Bartel, D. AGO1 homeostasis entails coexpression of MIR168 and AGO1 and preferential stabilization of AGO1.

38. Zhang, B., Pan, X., Cannon, C. H., Cobb, G. P. & Anderson, T. A. Conservation and divergence of plant miRNA genes.

37. Vaucheret, H., Mallory, A. & Bartel, D. AGO1 homeostasis entails coexpression of MIR168 and AGO1 and preferential stabilization of AGO1.

36. Juarez, M. T., Kui, J. S., Thomas, J., Heller, B. A. & Timmermans, M. C. microRNA-mediated repression of rolled leaf1 specifies maize leaf polarity.

35. Raman, S.

34. Ding, Q., Zeng, J. & He, X. Q. Deep sequencing on a genome-wide scale reveals diverse stage-specific microRNAs in cambium during dormancy-release induced by chilling in poplar.

33. Zhang, Y.

32. Srivastava, P. K., Moturu, T. R., Pandey, P., Baldwin, I. T. & Pandey, S. P. A comparison of performance of plant miRNA target prediction tools and the characterization of features for genome-wide target prediction.

31. Sunkar, R., Zhou, X., Yun, Z., Zhang, W. & Zhu, J. K. Identification of novel and candidate miRNAs in rice by high throughput sequencing and degradome analysis.

30. Sunkar, R., Zhou, X., Yun, Z., Zhang, W. & Zhu, J. K. Identification of novel and candidate miRNAs in rice by high throughput sequencing and degradome analysis.

29. Zhao, Y. T.

28. Zhu, Q. H.

27. Mi, S. et al. Small RNA sequencing reveals diverse stage-specific microRNAs in cambium during dormancy-release induced by chilling in poplar.

26. Zhang, Y. et al. Identification and characterization of cold-responsive microRNAs in tea plant (Camellia sinensis) and their targets using high-throughput sequencing and degradome analysis.

25. Zhang, Y. et al. Identification and characterization of cold-responsive microRNAs in tea plant (Camellia sinensis) and their targets using high-throughput sequencing and degradome analysis.

24. Zhang, Y. et al. Identification and characterization of cold-responsive microRNAs in tea plant (Camellia sinensis) and their targets using high-throughput sequencing and degradome analysis.

23. Zhang, Y. et al. Identification and characterization of cold-responsive microRNAs in tea plant (Camellia sinensis) and their targets using high-throughput sequencing and degradome analysis.

22. Zhang, Y. et al. Identification and characterization of cold-responsive microRNAs in tea plant (Camellia sinensis) and their targets using high-throughput sequencing and degradome analysis.

21. Zhang, Y. et al. Identification and characterization of cold-responsive microRNAs in tea plant (Camellia sinensis) and their targets using high-throughput sequencing and degradome analysis.

20. Kim, J.

19. Guo, H. S., Xie, Q., Fei, J. F. & Chua, N. H. MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate germination stages.

18. Guo, H. S., Xie, Q., Fei, J. F. & Chua, N. H. MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate germination stages.

17. Lauter, N., Kampani, A., Carlson, S., Goebel, M. & Moose, S. P. microRNA172 down-regulates glossy15 to promote vegetative phase change in maize.

16. Llave, C. & Carrington, J. C. Cleavage of scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA.

15. Chen, X. A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development.

14. Bartel, D. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function.

13. Zhang, Y. X.

12. Zhang, Y. X.

11. Zhang, Y.

10. Zhang, Y.

9. Zhang, Y.

8. Zhang, Y.

7. Zhang, Y.

6. Zhang, Y.

5. Zhang, Y.

4. Zhang, Y.

3. Zhang, Y.

2. Zhang, Y.

1. Zhang, Y.
53. Perl, M. ATP synthesis and utilization in the early stage of seed germination in relation to seed dormancy and quality. *Physiol. Plantarum.* 66, 177–182 (2010).
54. Chope, G. A., Cools, K., Terry, L. A., Hammond, J. P. & Thompson, A. J. Association of gene expression data with dormancy and sprout suppression in onion bulbs using a newly developed onion microarray. *Acta Horticulturae.* 969, 169–174 (2012).
55. Kumar, A. & Bhatia, S. C. Peptide markers for low temperature stress during seed germination in sunflower. *Biol. Plantarum.* 50, 81–86 (2006).
56. Nelson, D. C. *et al.* F-box protein MAX2 has dual roles in karrikin and strigolactone signaling in *Arabidopsis thaliana.* *Proc. Natl. Acad. Sci. USA* 108, 8897–8902 (2011).
57. Wu, G. & Poethig, R. S. Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target SPL3. *Development.* 133, 3539–3547 (2006).
58. Wang, J. W. *et al.* miRNA control of vegetative phase change in trees. *Plos Genet.* 7, e1002012, https://doi.org/10.1371/journal.pgen.1002012 (2011).
59. Meyers, B. C. *et al.* Criteria for annotation of plant MicroRNAs. *Plant Cell.* 20, 3186–3190 (2008).
60. Audic, S. & Claverie, J. M. The significance of digital gene expression profiles. *Genome Res.* 7, 986–995 (1997).
61. Man, M. Z., Wang, X. & Wang, Y. POWER_SAGE: comparing statistical tests for SAGE experiments. *Bioinformatics.* 16, 953–959 (2000).
62. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 25, 402–408 (2001).

**Acknowledgements**

This work was supported by grants from National Natural Science Foundation of China (31372104 and 31471908).

**Author Contributions**

Conceived and designed the experiments: Shupeng Gai. Designed, analyzed data, drafted and revised the manuscript: Yuxi Zhang and Shupeng Gai. Collected materials: Yanyan Wang. Analyzed the data: Yanyan Wang and Xuekai Gao. Gave valuable advice: Chunying Liu.

**Additional Information**

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-22415-5.

**Competing Interests:** The authors declare no competing interests.

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018