Transcriptome Comparison Reveals Key Candidate Genes Responsible for the Unusual Reblooming Trait in Tree Peonies

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Abstract

Tree peonies are important ornamental plants worldwide, but growing them can be frustrating due to their short and concentrated flowering period. Certain cultivars exhibit a reblooming trait that provides a valuable alternative for extending the flowering period. However, the genetic control of reblooming in tree peonies is not well understood. In this study, we compared the molecular properties and morphology of reblooming and non-reblooming tree peonies during the floral initiation and developmental processes. Using transcriptome sequencing technology, we generated 59,275 and 63,962 unigenes with a mean size of 698 bp and 699 bp from the two types of tree peonies, respectively, and identified eight differentially expressed genes that are involved in the floral pathways of Arabidopsis thaliana. These differentially regulated genes were verified through a detailed analysis of their expression pattern during the floral process by real time RT-PCR. From this combined analysis, we identified four genes, PsFT, PsVIN3, PsCO and PsGA20OX, which likely play important roles in the regulation of the reblooming process in tree peonies. These data constitute a valuable resource for the discovery of genes involved in flowering time and insights into the molecular mechanism of flowering to further accelerate the breeding of tree peonies and other perennial woody plants.

Introduction

The tree peony, which belongs to the genus Paeonia L. section Moutan DC. (Paeoniaceae), is one of the most important horticultural crops in the world due to its striking ornamental and medicinal values [1,2]. During its 1600 years of cultivation history, tree peonies were introduced from China to Japan and then to Europe and America; as result, these plants have exerted a tremendous impact and have introduced a flair for the dramatic to gardening [3,4]. In general, tree peonies flower in early May within a concentrated period of approximately twenty days. Given the commercial value of tree peonies, the achievement of a longer flowering period is the ultimate goal of breeders and growers [5]. Some cultivars have the ability to bloom twice during a given year, which provides a unique opportunity to lengthen the flowering period by exploiting the number rather than the length of the flowering cycles.

Reblooming is an important trait for a variety of horticultural crops that can extend their flowering period and increase their fruit production to thus produce flowers and fruit year-round. In an attempt to force reblooming in tree peonies, various cultivars have been selected, and certain practical techniques, including pruning, gibberellin (GA) treatment, defoliation, and moisture stress, have been considered for a number of years [6–8]. To date, tree peonies containing the reblooming trait have become popular in modern gardens and have brought tremendous ornamental and economic successes to China and Japan. A recent study showed that endogenous hormone and carbohydrate levels greatly influence the reblooming process in tree peonies [9]; however, the genetic control of reblooming in this population is not well understood. Molecular biology studies of tree peonies are lacking, although some progress has been made, including the discovery of some molecular marker [10,11], the analysis of the effect of chilling on dormancy release [12], and the isolation of the MADS-box genes [13,14].

Floral transition from a vegetative state to reproductive development in the shoot apical meristem (SAM) marks the beginning of floral initiation and determines the flowering time of a plant [15,16]. In perennial woody plants, including tree peonies, the juvenile phase of floral transition still poses a challenge for scientists [17]. However, the physiological and molecular genetics of the floral transition in herbaceous species have been extensively studied [18–20]. Four pathways controlling the floral transition have been organized in the model plant Arabidopsis thaliana. These are the photoperiod and vernalization pathways, which mediate the response to environmental cues, and the autonomous and GA pathways, which are mainly independent from external signals [21–23]. The understanding of the floral transition in annual model plants can provide a framework for the exploration this process in perennials.
RNA sequencing (RNA-Seq) is a transcriptome profiling approach that uses deep-sequencing technologies and thus provides a powerful tool for the analysis of species that lack reference genome information [24]. Recent advances in both DNA sequencing and assembly programs have made the low-cost construction of transcriptome datasets for non-model species feasible, which has speeded the process of functional gene discovery [25–27]. Many studies have used transcriptome comparisons to identify differentially expressed genes between distinctive plant phenotypes [28–31].

In this study, we performed transcriptome sequencing using reblooming and non-reblooming tree peonies to discover potential candidate genes involved in the control reblooming process. To the best of our knowledge, this study provides the first identification of potential flowering genes that are responsible for reblooming in tree peonies and thus serves as a seminal resource for the molecular control of flowering times and for further molecular breeding studies in tree peonies and other perennial plants.

**Materials and Methods**

**Plant materials and sample collection**

Two cultivars of tree peonies, namely reblooming *Paeonia loemoinei* ‘High Noon’ (HN) and non-reblooming *Paeonia suffruticosa* ‘Luo Yang Hong’ (LYH), were grown in the Jiu Feng Forestry Experiment Station of the Beijing Forestry University in China. The samples used for sequencing and expression analysis were collected using a binocular microscope to detect the development stage of the flower buds of HN and LYH, which were defined through morphological observation of the SAMs throughout the year using a scanning electron microscope (SEM).

Flowers, as well as developing buds, were harvested from HN and LYH for transcriptome sequencing in 2011. The shoot apexes of HN and LYH were collected every three days during floral induction in 2011 and used for quantitative RNA-Seq and real time RT-PCR verification. The following year, shoot apexes, including sprouting reblooming buds (usually big and with good nutrition) and non-reblooming buds within scales, were harvested from the same plants from the bud developmental process through the subsequent flowering for gene expression analysis by real time RT-PCR. These samples for real time RT-PCR were collected at approximately 10:00 AM of the sampling day. All of the samples were dissected (i.e., removal of scales and most of the leaves), immediately frozen in liquid nitrogen, and stored at −80°C until further processing.

**Scanning electron microscope (SEM)**

The floral induction and differentiation processes were observed through SEM. Prior to analysis, the SAM samples were fixed with 3% glutaraldehyde in 0.025 M sodium phosphate buffer (pH 6.8) at 4°C for 12 hour. The samples were then rinsed in the buffer, further fixed in 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.0) for several hours, and then dehydration through a graded ethanol series. The samples were then critical-point dried in liquid carbon dioxide. In many cases, the outer organs were dissected from the SAMs, and the mounted specimens were coated with gold and palladium (4:1) using a Technics Hummer V sputter coater. These studies were performed using an FEI Quanta 200 environmental scanning electron microscope (USA).

**RNA extraction and transcriptome sequencing**

The total RNA was isolated using an RN38-EASYspin Plus kit (Aidlab, China). The RNA was then treated with RNase-free D (Promega, USA), and its purity was assessed using a Nanodrop 2000C spectrophotometer (Thermo scientific, USA). After total RNA was collected, the poly (A) mRNA was isolated using Oligo(dT) beads and fragmented into short fragments. The first-strand cDNA was synthesized using random hexamer primers, and the second strand cDNA was synthesized using buffer, dNTPs, RNaseH and DNA polymerase I. These products were purified by agarose gel electrophoresis and enriched by PCR to create the final cDNA library. This library was commercially sequenced by the Beijing Genomics Institute in an Illumina HiSeq™ 2000 platform with paired-end sequencing using 90-bp reads to generate the raw sequences. These data are available at the NCBI Short Read Archive (http://trace.ncbi.nlm.nih.gov/Traces/sra_sub/sub.cgi, accession number: SRP026412 and SRP026299).

**De novo assembly and unigene functional annotation**

After all of adaptors and low-quality reads were removed from the raw sequences (N > 5%), transcriptome sequences were subjected to de novo assembly to form contigs using the short-reads assembling program Trinity [32]. The contigs were further connected to obtain unigenes using the TIGR clustering tool [33]. The unigenes were divided in to two classes: one was clusters with the prefix CL, and the other consisted of singletons with the prefix unigene. BLASTX alignment and ESTScan was used to predict the coding regions and to determine the sequence direction [34].

For the functional annotation of unigenes, the remaining sequences that could putatively encode proteins were searched against the following protein databases: NCBI non-redundant protein database (NR), Swiss-Prot protein database, Kyoto Encyclopedia of Genes and Genomes (KEGG) database, Gene ontology (GO) database using BLASTX search, and nucleotide database (NT) by BLASTN with e-value less than 0.00001. Based on the NR annotation, the Blast2GO program was used to obtain GO annotation of unigenes [35]. The GO functional classification was analyzed using WEGO software [36].

**RNA quantification and screening of differentially expressed genes**

RNA-Seq quantification was performed during floral induction using the same protocol that was used for the transcriptome sequencing except that the RNA-Seq quantification involves single-end sequencing with 50-bp reads. All of the clean reads were mapped to the reference transcriptome sequences (all unigenes) using the SOAPAligner/soap2 program, and the final alignment contained no more than two mismatched base pairs [37].

The gene expression level was calculated using the Reads Per Kilobase per Million mapped reads (RPKM) method [38]. Based on “the significance of digital gene expression profiles”, differentially expressed genes (DEGs) between samples and their corresponding P-value were determined using methods described by Audic and Claverie [39]. The False Discover Rate (FDR) was used determine the threshold of the P-value in multiple tests. We used $FDR \leq 0.001$ and the absolute value of log2Ratio $\geq 1$ as the threshold to judge the significance of the gene expression differences [40].
Real time RT-PCR verification and expression analysis

The RNA extracted from shoot apex from all of the samples during different floral initiations in HN and LYH in 2011 and 2012 was used for the reverse transcriptase (RT) reaction. The eight specific primer pairs used for real time RT-PCR analyses are shown in Table S1. The PCR was performed on a Mini option Real-Time PCR instrument (Bio-Rad, USA) using 20 μl Taq™ (TaKaRa, Japan). The PCR program was initiated at 95°C for 30 s and was followed by 40 cycles of 95°C for 3 s, 55°C for 30 s, and 72°C for 30 s. A melting curve analysis was then performed for each reaction. The expression levels of the candidate genes were calculated using the $2^{-ΔΔCt}$ method and normalized to the reference gene Ubiquitin and GAPDH [41]. The real time RT-PCR reaction was performed in three biological replicates, and three technical repetitions were performed for each replicate. Expression differences between samples were analyzed by analysis of variance (ANOVA) using SPSS 18.0 software.

Results

Morphological description of floral initiation in HN and LYH

The ‘High Noon’ (HN) cultivar of tree peonies has been shown to have a stable reblooming trait under cultivation in different countries. As a control, the non-reblooming ‘Luo Yang Hong’ (LYH) cultivar, which is one of the most popular and widely cultivated species in China, was used. Under natural conditions after the winter period, axillary buds give rise to a shoot that is terminated by a flower. Then, in most tree peonies, such as LYH, secondary shoots and annual terminal shoots undergo floral induction in June and then floral differentiation surrounded by bud scales until entering the dormant winter season (Figure 1 A-D). The corresponding histological changes that occur during floral induction and differentiation in LYH were assessed using SEM and are shown in Figure 1 E-H.

The flowering in HN occurs later than in LYH. In this cultivar, floral induction generally takes place in August and followed by floral differentiation within floral buds and then dormancy (Figure 1 M-N). However, some annual terminal shoots and secondary shoots in HN can sprout early in the year; these tend to exhibit rapid floral induction and differentiation and rebloom within a year (Figure 1 J-L). The histological observations during the floral process in HN are shown in Figure 1 O-T. For Paeonia, the bracts initiation reveals the end of the vegetative stage and marks the floral transition [42,43]. The results clearly show one floral transition in LYH (Figure 1 F) and two floral transitions in HN: one transition occurred in the growth buds and resulted in reblooming (Figure 1 P), and the other transition occurred within bud scales to introduce the next spring flowering (Figure 1 R).

The morphological observations ascertained that there is a distinct difference between reblooming and non-reblooming tree peonies and demonstrated that early floral transition and rapid differentiation are critical events in the reblooming cultivar. Therefore, we performed floral transcriptome sequencing between HN and LYH cultivars to further understand these differences at the molecular level.

Transcriptome sequencing of HN and LYH and de novo assembly

We constructed cDNA libraries for both HN and LYH. Total of 55,245,430 and 57,483,678 raw 90-bp reads were separately generated on an Illumina HiSeq™ 2000 instrument (Table 1). The primer and adaptor sequences were removed to generate clean reads, which were then subjected to cluster and assembly analysis. The software assembled 150,407 and 132,499 contigs with a mean length of 320 bp and 334 bp for HN and LYH, respectively, and obtained 63,962 and 59,275 unigenes with a mean size of 699 bp and 695bp, respectively (Table 2). Altogether, 84,919 unigenes were clustered from the two cultivars, and 33,500 and 51,419 of these clusters and singletons, respectively (Table 2). The size distribution of the assembled contigs and unigenes is presented in Table S2.

Transcriptome functional annotation and comparison

To identify the putative functions of the assembled unigenes in HN and LYH, we searched for sequence similarities in five public databases: NR, Swiss-Prot, KEGG, GO database using the BLASTX search and NT by BLASTN. In total, 36,821 of the 63,962 (57.5%) in HN and 36,475 of the 59,275 (61.5%) unigenes in LYH have been annotated in the public databases (Table 3). Of the BLAST hits, most of the unigenes (96.8% in HN and 96.7% in LYH) were annotated by the NR database; the annotated unigenes are listed in Table S3.

To understand the differences in the floral initiation process between HN and LYH, their transcription were compared. The GO analysis showed that the distribution of gene functions for the cDNA sequences from HN and LYH were similar in three main categories: biological processes, cellular components, and molecular functions (Figure 2). Among these categories, the cell, cell junction, binding, catalytic activity, organelle, metabolic process and cell process were the groups with the highest representation of genes from both cultivars.

Functional classification and pathway assignments for HN and LYH were performed through KEGG analyses. A total of 19,135 and 19,257 matched sequences of HN and LYH, respectively, were assigned to 127 KEGG pathways. The most represented pathways in HN and LYH are the metabolic pathways (20.56% and 21.13%, respectively), the biosynthesis of secondary metabolites (10.1% and 9.64%, respectively), plant-pathogen interaction (5.99% and 5.55%, respectively), and plant hormone signal transduction pathways (5.38% and 5.16%, respectively; Table S4).

This transcriptome annotation between HN and LYH provides a valuable resource for gene discovery and functional analysis in tree peonies.

Screening and analysis of differentially expressed genes (DEGs) through quantitative RNA-Seq

The morphological analysis demonstrated that the early floral transition in HN is the primary event that characterizes reblooming. To determine potential genes involved in reblooming induction, we compared the gene expression profiles of HN and LYH during floral transition. Floral induction in tree peonies is defined as the stage when leaf primordial transitions to bract primordial [44]. The exact sampling period in the present work was from 5 June to 25 June 2011 for LYH and for reblooming induction in HN and from 1 August to 25 August 2011 for normal floral induction in HN. Subsequently, the samples collected during floral induction were used to construct three cDNA libraries, namely floral induction in LYH (L), normal floral induction in HN (H1), and reblooming induction in HN (H2), for quantitative RNA-Seq analysis. We generated 12,488,008, 12,380315 and 12,041,487 50-bp reads through Illumina sequencing from the L, H1 and H2 libraries, respectively (Table S5). After removing the adaptor and low-quantity raw reads, we mapped the clean reads of these three libraries to the transcriptome database, which contains 84,919 unigenes. More than 80% of the clean reads in the induction stages were mapped to reference transcriptome sequences, and 58.43%, 56.29%, and 58.37% unique matches
were found for these three stages (Table S5). The differentially expressed genes (DEGs) were then identified from the three floral induction cDNA libraries. As expected, the majority of gene expression changes occurred between L and H1/H2, and slightly more up-regulated genes were observed compared with down-regulated genes among the groups (Figure 3 and Table S6, S7, S8).

GO and KEGG assignments were performed to clarify the functions of the DEGs in the three cDNA libraries during floral induction. Fewer DEGs were classified into the three major GO categories (cellular component, molecular function, and biological process) in L-VS-H1 compared with H1-VS-H2 and L-VS-H2 (Figure 4). In the cellular component category, the ‘intracellular
Table 1. Summary of HN and LYH transcriptome sequencing

| Sample | Total raw reads | Total clean reads | Total clean nucleotides (nt) | Q20 percentage | N percentage | GC percentage |
|--------|----------------|------------------|-------------------------------|----------------|--------------|--------------|
| HN     | 55,245,430     | 51,411,776       | 4,627,059,840                | 97.23%         | 0.00%        | 45.87%       |
| LYH    | 57,483,678     | 54,163,770       | 4,874,739,300                | 97.99%         | 0.00%        | 46.13%       |

Candidate genes in the floral pathway from DEGs

Based on the functional annotation, all of the unigenes were analyzed through BLASTX searches, and 21 known Arabidopsis homologous genes involved in four floral pathways were identified (Table S10). Of these 21 genes, eight genes were differentially expressed between the three floral induction patterns and may thus be associated with reblooming induction (Table 4).

The analysis of the autonomous pathway showed that most of the homologous genes of Arabidopsis were present in tree peonies [45]. The expression of these genes, although slightly variable, exhibited no significant difference among the three floral inductions (Table S10).

An analysis of the photoperiod pathway using the homologous genes in Arabidopsis [46] demonstrated that certain photoperiod response genes exhibited a higher expression level in HN compared with LYH, although not all of the genes were significantly differentially expressed (Table S10). CO (CONSTANS) [47] and GI (GIBBERELLIN INSENSITIVE DWARF 1) [48], which are two important photoperiod genes, showed approximately seven- and four-fold higher expression levels in H1 and H2 than in L (Table 4). In the vernalization pathway [49], only two homologous genes, namely FRI (FRIGIDA) [50] and VIN3 (VERNALIZATION INSENSITIVE 3) [51], were found. PsFRI, which is a floral repressor, and PsVIN3, which is a flowering enhancer, presented converse expression in H2 compared to H1 and L (Table 4). Of the four homologous genes found in the GA-signaling pathway [52], GA20OX (GIBBERELLIN 20OXIDASE), which is a key gene responsible for active GA synthesis [33], and GID1 (GIBBERELLA INSENSITIVE DWARF 1), which is an active GA receptor [54], exhibited similar down-regulation in H2 compared to H1 and L (Table 4).

In addition to the four floral pathways, we searched for homologs for the floral pathway integrator SOC1 (SUPPRESSOR OF CONSTANS OVEREXPRESSION 1), FT (FLOWERING LOCUS T), LFY (LEAFY), and the meristem identity gene AP1 (APETALA 1) in Arabidopsis (Table S10) [55,56]. Through this analysis, the SOC1 [57] and FT [58] homologous genes were shown to be differentially expressed between the different floral inductions (Table 4).

In general, eight DEGs, namely PsCO, PsGI, PsFRI, PsVIN3, PsGA20OX, PsGID1, PsSOC1 and PsFT, were found to be candidate genes associated with reblooming induction. To date, no function has been reported for any of these genes in tree peonies, although two of these genes, PsGA20OX (GenBank ID: ABQ52488.1) and PsAP1 (GenBank ID: ADM21461.1), are registered in the NCBI with unknown gene function.

Expression analysis of candidate genes during the flowering process in HN and LYH

To verify the accurateness of the RNA-Seq data (Figure 5 A), we validated the candidate DEGs through real time RT-PCR. As shown in Figure 5 B, the expression levels of these genes are varied among the three floral induction stages, and the expression pattern of these genes was similar to those obtained through RNA-Seq, which confirms the validity of the RNA-Seq data. A Pearson correlation analysis between the gene expression levels measured by real time RT-PCR and RNA-Seq showed a highly significant correlation (correlation coefficient R = 0.785, P < 0.01), which indicates good reproducibility between the transcript abundance assayed by RNA-Seq and the expression profile revealed by real time RT-PCR (Figure 5 C).

Through our study of floral induction, we discovered eight differentially expressed genes that are associated with reblooming. To further confirm the role of these genes in reblooming, we

Table 2. Summary of HN and LYH transcriptome assembly

| Sample | Total number | Total length (nt) | Mean length (bp) | N50 | Clusters | Singleton |
|--------|--------------|------------------|-----------------|-----|----------|-----------|
| Contig | HN           | 150,407          | 48,120,963      | 320 | 590      |           |
| Contig | LYH          | 132,499          | 44,276,926      | 334 | 624      |           |
| Unigene| HN           | 63,962           | 44,707,077      | 699 | 1,077    | 27,452    |
| Unigene| LYH          | 59,275           | 41,380,872      | 698 | 1,075    | 19,288    |
| All Unigene | 84,919 | 58,746,795      | 692             | 1,093 | 33,500 | 51,419 |

Table 3. Summary of HN and LYH transcriptome assembly

| Sample | Total number | Total length (nt) | Mean length (bp) | N50 | Clusters | Singleton |
|--------|--------------|------------------|-----------------|-----|----------|-----------|
| Contig | HN           | 150,407          | 48,120,963      | 320 | 590      |           |
| Contig | LYH          | 132,499          | 44,276,926      | 334 | 624      |           |
| Unigene| HN           | 63,962           | 44,707,077      | 699 | 1,077    | 27,452    |
| Unigene| LYH          | 59,275           | 41,380,872      | 698 | 1,075    | 19,288    |
| All Unigene | 84,919 | 58,746,795    | 692             | 1,093 | 33,500 | 51,419 |
analyzed their change in gene expression during floral initiation in HN and LYH by real time RT-PCR in 2012–2013 (Figure 6).

The analysis of the floral development process showed that the PsFT expression was low during induction and then increased significantly during differentiation in both HN and LYH (Figure 6 A). The statistical analysis showed that PsFT decreased significantly during the two floral inductions (H2 and H1) in HN and only once (L) in LYH (Figure 6 A). The PsVIN3 gene presented a
high expression level during reblooming differentiation in July and decreased during normal differentiation in HN (Figure 6 B). The PsCO gene exhibited a high expression level during reblooming induction and normal differentiation in HN (Figure 6 C). Similarly, the PsVIN3 and PsCO genes presented the same expression pattern as PsFT in LYH, which was significantly accumulated during differentiation (Figure 6 A, B, C). The PsGA20OX gene showed fluctuation during the floral process in HN and LYH and accumulated during differentiation (Figure 6 D).

Certain genes, including PsSOC1, PsFRI and PsGID1, exhibited a different expression pattern with high expression during the normal floral transition in HN (Figure 6, E, F, G). In contrast to PsFT, the expression of PsSOC1 gene was high during induction, decreased during differentiation, and increased again during bud dormancy; this pattern was consistent in the three floral initiation periods (Figure 6 E). The expression of the PsFRI gene, which is
the only floral repressor in our list of candidate DEGs, was high during floral induction and differentiation and decreased significantly after bud burst in LYH; in contrast, this gene exhibited a significant low expression during reblooming differentiation, and its level was accumulated during bud dormancy in HN (Figure 6F). The \textit{PsGID1} gene was accumulated early during the differentiation processes and decreased from October to February in both HN and LYH (Figure 6G).

The \textit{PsGI} gene demonstrated a similar expression pattern as \textit{PsCO} throughout the floral developmental process in HN but exhibited higher accumulation during floral differentiation in 30 August in LYH compared with HN (Figure 6C, H).

Discussion

Reblooming in tree peonies: a distinctive floral initiation

Reblooming is a common phenomenon in horticultural crops and is one way to efficiently extend the flowering period. There are two conditions for reblooming during the same year for perennial plants: one can flower continuously during the favorable season, and the other may only have a second bloom later in the season [59]. Most horticultural crops, such as some fruit trees, belong to the latter due to the break out of resting buds. In this study, we analyzed the morphological modifications that occur during the reblooming process in HN. The results showed that the reblooming in HN may be distinct from other reblooming processes. First, two floral transitions during two concentrated periods take place in HN: the first transition in June results in reblooming, and the second transition in August introduces the flowering for the following spring. However, in some fruit trees, reblooming has only one floral transition, and some environment conditions induce buds to break and to thus flower earlier than dormancy buds [60]. Second, floral induction and differentiation during reblooming in HN are accompanied by bud sprouting and growth (Fig. 1J, K, P, Q). In contrast, bud breakage usually occurs in non-sprouting dormancy buds that have partly or completely differentiated their floral primordial [61].

The timing of plant flowering varies greatly by genotype and is highly dependent on genetic and environmental interactions. In roses, continuous flowering is self-inductive and is not dependent on environmental control, whereas the floral induction of single flowering plants is dependent on environmental control [62]. In fact, conflicting reports state that woodland strawberries have either autonomous control of flowering [63] or long-day rather than autonomous control of continuous flowering [64]. The following genes play key roles in the control of continuous flowering in roses and strawberries: the \textit{TFL1} homologue \textit{KSN} [59], \textit{RECURRENT BLOOMING (RB)} and \textit{SEASONAL FLOWERING LOCUS (SFL)} in roses and strawberries [65,66]. However, the genes identified in rose and strawberry plants do not appear to be

| Table 4. Putative candidate genes of the DEGs associated with floral induction in tree peonies |

| Tree peony gene | Arabidopsis ID | Unigene ID | % identity with homologs | RPMK of H1 H1-VS-H2 FDR | RPMK of H2 L-VS-H1 FDR | RPMK of L L-VS-H2 FDR |
|-----------------|----------------|------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Photoperiod pathway | | | | | | |
| \textit{PsCO} | NP_568863.1 | CL8074.Contig2_All | 47 | 17.3 | 12.5 | 0.09 |
| | | | | -0.47 | 7.55 | 7.08 |
| | | | | 0.027 | 8.65E-54 | 2.86E-38 |
| \textit{PsGI} | NP_564180.1 | CL9113.Contig1_All | 75 | 2.24 | 2.19 | 0.13 |
| | | | | -0.03 | 4.04 | 4.0 |
| | | | | 0.98 | 1.32E-14 | 3.14E-14 |
| Vernalization pathway | | | | | | |
| \textit{PsFRI} | NP_850923.1 | CL10369.Contig1_All | 63 | 48.3 | 23.8 | 85.7 |
| | | | | -1.02 | -0.83 | -1.85 |
| | | | | 9.45E-29 | 6.94E-36 | 1.1E-124 |
| \textit{PsVIN3} | NP_200548.2 | Unigene3778_All | 41 | 12.76 | 21.5 | 76.8 |
| | | | | 0.75 | 0.73 | 1.48 |
| | | | | 6.16E-07 | 0.00017 | 1.97E-20 |
| GA pathway | | | | | | |
| \textit{PsGA20OX} | NP_194272.1 | Unigene3745_All | 52 | 7.81 | 3.47 | 9.97 |
| | | | | -1.17 | -0.35 | -1.52 |
| | | | | 0.00067 | 0.21 | 1.71E-07 |
| \textit{PsGID1} | NP_187163.1 | Unigene2987_All | 81 | 243.4 | 67.8 | 204.7 |
| | | | | -1.84 | 0.25 | -1.6 |
| | | | | 0 | 2.23E-10 | 5.26E-211 |
| Floral integrators | | | | | | |
| \textit{PsSOC1} | NP_182090.1 | Unigene15356_All | 61 | 19.1 | 50.6 | 48.2 |
| | | | | 1.4 | -1.33 | 0.067 |
| | | | | 2.64E-22 | 5.68E-20 | 0.63 |
| \textit{PsFT} | NP_176726.1 | Unigene5093_All | 78 | 1.21 | 6.73 | 0.31 |
| | | | | 2.47 | 1.95 | 4.42 |
| | | | | 4.51E-06 | 0.15 | 3.14E-10 |

H1-VS-H2: log2 gene expression level in H2 compared to H1.
A FDR (false discovery rate) < 0.001 indicates a significant difference.
Note: percent identity was calculated by comparing the \textit{Arabidopsis} and tree peony sequences at the amino acid level.

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the candidate genes for tree peonies, which indicate that separate mechanisms control reblooming in these different plant species.

A comprehensive floral transcriptome of tree peonies
To identify genes that are involved in the reblooming process in tree peonies, we sequenced the floral transcriptomes from reblooming HN and non-reblooming LYH. We generated a total of 84,919 unigenes with mean reads of 692 bp, which corresponds to a 3.5-fold higher number of unigenes compared with the transcriptome of *Paeonia ostii* 'Feng Dan' for which 23,652 contigs/singleton were generated from dormancy buds, which accounts for 15,284 contigs/singleton with an average mean read of 738.9 bp using the Roche 454 GS FLX platform [67]. More than 50% of all of the unigenes were annotated using public databases with key homologous genes involved in the four floral pathways of *Arabidopsis*. These floral transcriptomes of both cultivars provide more comprehensive information and gene resources for the study of the floral transition and development in tree peonies and might facilitate further investigations into the molecular mechanism of flowering in this important crop.

Candidate genes for the control of reblooming in tree peonies

Based on transcriptome and gene expression analyses of the floral process in HN and LYH, we identified genes that are differentially expressed in these floral pathways and demonstrated that these may play a role in reblooming.

Flowering is controlled by a complex network of signaling pathways. One environmental cue is light. In *Arabidopsis*, flowering is induced in long-day conditions by the *CO* gene, which up-regulates the expression of the *FT* gene [68]. However, no previous reports have described the influence of the photoperiod on flowering in tree peonies. In this study, the *PsCO* gene was accumulated during reblooming induction and exhibited a higher expression level in HN compared with LYH (Figure 6 C, H). The up-regulation of the expression of this gene during reblooming induction coincided with the bud sprouting and outgrowth that occurred in HN in June. The KEGG pathway analysis of the DEGs during floral induction showed that photosynthesis was significant enriched in H1-VS-H2 (3.1% of DEGs) and L-VS-H2 (0.87% DEGs) (Table S9). Therefore, compared to normal
flowering, we conclude that light may have a stronger effect on induction of reblooming and bud growth. Given that the constitutive overexpression of CO causes rapid flowering even in non-inductive conditions [69], the fact that the PsCO gene is associated with reblooming in our study is very interesting and warrants further investigation.

In perennial woody plants, such as tree peonies, chilling is normally required for dormancy release prior to flowering, and this process is similar to vernalization in Arabidopsis, where flowering is promoted through exposure to cold temperatures [70]. However, HN blooms directly without cold vernalization (Figure 1 I-L). In this study, the high expression of the vernalization gene PVIN3 and the low expression of PsFRI during floral differentiation in 10 July is likely responsible for the direct reblooming in HN. VNZ, which is a key gene that responds to vernalization conditions in Arabidopsis, is induced by low temperatures and repressed upon a return to warmer temperature [71,72]. In HN, PVIN3 was up-regulated during reblooming induction on 20 June and was abundantly accumulated during reblooming differentiation on 10 July (Figure 6 E). Remarkably, the expression pattern of PVIN3 was similar to PFT in both cultivars during floral development (Figure 6 A, B). These results suggest that both PVIN3 and PFT most likely play important roles in the promotion of reblooming.

Considerable physiological and molecular analyses have shown that GA plays an important role in the control of floral transition, and its role may be species-specific [73]. In Arabidopsis, GA contributes to the floral pathway through the promotion of floral induction; in contrast, GA generally inhibits flowering in woody angiosperms [17,74,75]. In this study, a low expression of PPGA20OX and PGI was detected in all three floral inductions (Figure 6 D, G), which suggests that GA might play a role in tree peonies that is similar to its role in the inhibition floral induction in other woody plants. PPGA20OX exhibited very low expression during reblooming induction but was rapidly increased afterward to reach peak abundance (Figure 6 D), which indicates that a low expression of GA may induce reblooming and that a high expression of GA may promote reblooming differentiation. This finding is consistent with the results from other studies, which have demonstrated that exogenous application of GA3 to flower buds can help promote flowering in tree peonies. In contrast to PPGA20OX, PGID1 was accumulated during normal floral transition in HN (Figure 6 D, G), which is similar to the feedback regulation of GID1 through the GA signaling pathway in rice and Arabidopsis [76,77]. The expression of the PPGA20OX gene fluctuated similarly to the change in the GA3 pattern during floral initiation, as was previously reported in tree peonies [9,78]. This finding leads us to hypothesize that PPGA20OX plays an important role in reblooming in tree peonies that is mediated through the GA pathway.

Floral pathway integrators incorporate multiple floral pathways to determine the exact flowering time [79]. In this study, the expression level of the PsSOC1 gene was initially high and then decreased during floral initiation (Figure 6 E), which indicates that PsSOC1 might play a role in the promotion of floral transition but has no effect on floral organogenesis in tree peonies. In additional, the level of PsSOC1 increased significantly again in 21 December, and this change corresponded to a change in the expression of PsFRI, which suggests that PsSOC1 might be associated with chilling requirement and bud dormancy break [80]. FT, which is as a major component of florigen, is involved in the regulation of the floral transition in all angiosperms examined to date [81]. In trees, FT also controls seasonal growth cessation and dormancy [82,83]. PFT, which was found to be accumulated through the floral process, demonstrates a conserved role in the promotion of flowering in tree peonies. Notably, the expression of PFT was up-regulated before reblooming, and this increase coincided with the accumulation of PVIN3, PsCO and PPGA20OX in the sprouting buds (Figure 6 A, B, C, D), which suggests that PFT might be associated with bud growth and that PFT promotes reblooming by integrating different endogenous and environmental signals from the vernalization, photoperiod and GA pathways.

Conclusions

In conclusion, this study provides the first set of comprehensive floral transcriptome data in tree peonies. The transcriptome comparison between reblooming and non-reblooming tree peonies revealed eight DEGs as potential candidates responsible for reblooming. Detailed expression analyses of these genes during floral transition demonstrated that four DEGs, namely PFT, PVIN3, PsCO, and PPGA20OX might be responsible for reblooming in tree peonies. These findings offer the first insights into the potential mechanisms underlying flowering and reblooming in tree peonies.

Supporting Information

Table S1 Primers for real time RT-PCR analysis. (DOC)

Table S2 Overview of HN and LYH transcriptome sequencing and assembly. (A) Size distribution of Illumina sequencing contigs. (B) Size distribution of Illumina sequencing unigenes after paired-end gap filling. (XLS)
44. Wang ZZ, Zhang YX (1987) Studies on morphogenesis and life cycle of the flower bud of tree peony (Paeonia suffruticosa Andr.). Journal of Shandong Agricultural University 3: 9–16.

45. Simpson GG (2004) The autonomous pathway: epigenetic and post-transcriptional gene regulation in the control of Arabidopsis flowering time. Curr Opin Plant Biol 7: 570–574.

46. Mouradov A, Cremer F, Coupland G (2002) Control of flowering time: interacting pathways as a basis for diversity. Plant Cell 14 Suppl: S111–S130.

47. Suarez-Lopez P, Wheatley K, Robson F, Onouchi H, Valverde F, et al. (2001) Controlling the circadian clock and the control of flowering in Arabidopsis. Nature 410: 1116–1120.

48. Mizoguchi T, Wright L, Fujiwara S, Cremer F, Lee K, et al. (2005) Distinct roles of GID1α/1β in promoting flowering and regulating circadian rhythms in Arabidopsis. Plant Cell 17: 2255–2270.

49. Amaisono RM (2004) Vernalization, competence, and the epigenetic memory of winter. Plant Cell 16: 2553–2559.

50. Johanson U, West J, Lister C, Michaels S, Amaisono R, et al. (2000) Molecular analysis of FRIGIDA, a major determinant of natural variation in Arabidopsis flowering time. Science 290: 344–347.

51. Sung S, Amaisono RM (2004) Vernalization and flowering pathways in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. Nature 427: 159–164.

52. Sun TP, Gubler F (2004) Molecular mechanism of gibberellin signaling in plants. Annu Rev Plant Biol 55: 197–223.

53. Sakamoto T, Miura K, Itoh H, Tatsumi T, Ueguchi-Tanaka M, et al. (2004) An overview of gibberellin metabolism enzyme genes and their related mutants in rice. Plant Physiol 134: 1642–1653.

54. Hirano K, Ueguchi-Tanaka M, Matsuoka M (2008) GID1-mediated gibberellin signaling in plants. Trends Plant Sci 13: 278–284.

55. Sun TP, Gubler F (2004) Molecular mechanism of gibberellin signaling in plants. Annu Rev Plant Biol 55: 197–223.

56. Parcy F, Wright L, Fujiwara S, Cremer F, Lee K, et al. (2005) Distinct roles of GID1α/1β in promoting flowering and regulating circadian rhythms in Arabidopsis. Plant Cell 17: 2255–2270.

57. Griffiths J, Murase K, Rieu I, Zentella R, Zhang ZL, et al. (2006) Genetic analysis of FRIGIDA, a MADS-box gene closely related to Arabidopsis FRIGIDA. Plant Cell 18: 3399–3414.

58. Zheng GS, Gai SP, Gai WI (2009) Changes of endogenous hormones during dormancy release by chilling in tree peony. Scientia Silvae Sinicae 2: 48–52.

59. Simpson GG, Dean C (2002) Arabidopsis, the Rosetta stone of flowering time? Science 296: 285–289.

60. Griffiths J, Murase K, Rieu I, Zentella R, Zhang ZL, et al. (2006) Genetic characterization and functional analysis of the GID1 gibberellin receptors in Arabidopsis. Plant Physiol 139: 3069–3074.

61. Hartweck LM, Alizarzki NE (2006) Rice GIBBERELLIN INSENSITIVE DWARF1 is a gibberellin receptor that illuminates and raises questions about GA signaling. Plant Cell 18: 791–800.

62. Dennis ES, Hellwell CA, Peacock WJ (2006) Vernalization: spring into flowering. Dev Cell 11: 1–2.

63. Matea-Gottgens E, Helliwell CA, Peacock WJ (2006) Vernalization: spring into flowering. Dev Cell 11: 1–2.

64. Horvath D (2009) Common mechanisms regulate flowering and dormancy. Plant Cell 21: 4064–4077.

65. Blazquez MA, Green R, Nilsson O, Sussman MR, Weigel D (1998) Fine-scale genetic linkage analysis of the flowering pathway in Arabidopsis. Nature 401: 1116–1120.

66. Semeniuk P (1971) Inheritance of recurrent blooming in Rosa chinensis. Journal of Heredity 62: 203–204.

67. Battey NH, Miere PI, Tehranifar A, Cekic C, Taylor S, et al. (1998) Genetic and environmental control of flowering in strawberry. Genetic and Environmental Manipulation of Horticultural Crops. CABI, Wallingford, UK, pp 111–131.

68. Sonstely A, Heide OM (2007) Long-day control of flowering in everbearing strawberries. J. Hort. Sci. Biotechnol 82: 875–884.

69. Albuani MC, Battey NH, Wilkinson MJ (2004) The development of ISSR-derived SCAR markers around the seasonal flowering locus (SFL) in Fragaria vesca. Theor Appl Genet 109: 571–579.

70. Allen BC, Battey NH, Wilkinson MJ (2004) The development of ISSR-derived SCAR markers around the seasonal flowering locus (SFL) in Fragaria vesca. Theor Appl Genet 109: 571–579.

71. Foucher F, Chevalier M, Corre C, Soufflet-Freslon V, Legeai F, et al. (2006) Developmental stage-dependent expression of a MADS-box gene closely related to Arabidopsis FRIGIDA. Plant Cell 18: 3399–3414.

72. Hartweck LM, Alizarzki NE (2006) Rice GIBBERELLIN INSENSITIVE DWARF1 is a gibberellin receptor that illuminates and raises questions about GA signaling. Plant Cell 18: 278–282.