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RNA-Seq-based transcriptome analysis of dormant flower buds of Chinese cherry (*Prunus pseudocerasus*)

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ABSTRACT

Bud dormancy is a critical biological process allowing Chinese cherry (*Prunus pseudocerasus*) to survive in winter. Due to the lake of genomic information, molecular mechanisms triggering endodormancy release in flower buds have remained unclear. Hence, we used Illumina RNA-Seq technology to carry out de novo transcriptome assembly and digital gene expression profiling of flower buds. Approximately 47 million clean reads were assembled into 50,604 sequences with an average length of 837 bp. A total of 37,650 unigene sequences were successfully annotated. 128 pathways were annotated by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and metabolic, biosynthesis of second metabolite and plant hormone signal transduction accounted for higher percentage in flower bud. In critical period of endodormancy release, 1644, significantly differentially expressed genes (DEGs) were identified from expression profile. DEGs related to oxidoreductase activity were especially abundant in Gene Ontology (GO) molecular function category. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis demonstrated that DEGs were involved in various metabolic processes, including phytohormone metabolism. Quantitative real-time PCR (qRT-PCR) analysis indicated that levels of DEGs for abscisic acid and gibberellin biosynthesis decreased while the abundance of DEGs encoding their degradation enzymes increased and *GID1* was down-regulated. Concomitant with endodormancy release, MADS-box transcription factors including *P. pseudocerasus* dormancy-associated MADS-box (*PpcDAM*), *Agamous-like2*, and *APETALA3-like* genes, shown remarkably epigenetic roles. The newly generated transcriptome and gene expression profiling data provide valuable genetic information for revealing transcriptomic variation during bud dormancy in Chinese cherry. The uncovered data should be useful for future studies of bud dormancy in *Prunus* fruit trees lacking genomic information.

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1. Introduction

Flower bud dormancy in perennial deciduous trees is an adaptive mechanism to avoid injury in the winter. There are three main types of dormancy: paradormancy, endodormancy, and ecodormancy (Lang et al., 1987). Endodormancy is thought to be regulated by internal growth inhibitors. Even under favorable conditions, endodormant buds cannot initiate growth unless exposed to a specific duration of cold temperature, referred to as the chilling requirement. Once the chilling requirement has been satisfied, endodormancy is released, and endodormant buds gradually shift to the ecodormant state occurring under unfavorable environmental conditions (cold or drought). Dormancy establishment and release have been extensively studied in perennial deciduous trees.

Chinese cherry (*Prunus pseudocerasus*) is one of the most important and popular temperate fruit species in China. Like other perennial deciduous fruit trees, Chinese cherry trees undergo characteristic dormant periods during their annual growth cycles. In Zhejiang Province, China, cherry flower buds fulfill their chilling requirement and shift to the ecodormant state in late December, and then bloom the following spring. During this period, insufficient cold accumulation will result in non-uniform flowering, which subsequently reduces fruit set and fruit production. Investigation of the genetic factors underlying endodormancy establishment and release in cherry would enable artificial control of

Abbreviations: RNA-Seq, RNA sequencing; DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; qRT-PCR, quantitative real-time PCR; DAM, dormancy-associated MADS-box; C.U., chilling units; BGI, Beijing Genome Institute; RPKM, reads per kb per million; FDR, false discovery rate; EST, expressed sequence tag; COG, Clusters of Orthologous Groups of proteins; GST, glutathione S-transferase; ABA, abscisic acid; NCED, 9-cisepoxycarotenoid dioxygenase; PP2C, type 2C protein phosphatases; GA, Gibberellic acid; GA20ox, GA20 oxidase; GA2ox, GA2 oxidase; GA3ox, GA3 oxidase; AUX1, auxin influx carrier; ARF, auxin response factor; PTF, plant transcription factors; AP1-like, APETALA1-like; PI-like, PISTILLATA-like; AG-like, AGAMOUS-like; AP3-like, APETALA3-like; SHP-like, SHATTERPROOF-like; SEP-like, SEPALLATA-like; FT, FLOWERING LOCUS T; FLC, FLOWERING LOCUS C; SOC1, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1; BR6ox1, brassinosteroid-6-oxidase 1.

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endodormancy through cultural practices or rapid breeding techniques. Most studies of dormancy in Chinese cherry, a non-model plant lacking genomic information, have focused mainly on chilling requirements (Alburquerque et al., 2008; Crook and Black, 2010; Guak and Neilsen, 2013; Castede et al., 2014), with few reports addressing dormancy at the molecular level in this species.

Dormancy has been studied at the molecular level in various deciduous fruit trees. In these investigations, dormancy-associated MADS-box (DAM) genes have been isolated and their expression patterns studied during dormancy. Currently identified DAM genes are *DAM1*–*DAM6* in the genomes of peach (*Prunus persica*) (Bielenberg et al., 2008; Jimenez et al., 2009) and Japanese apricot (*Prunus mume*) (Sasaki et al., 2011), *MADS13-1*, *MADS13-2*, and *MADS13-3* in Japanese pear (*Pyrus pyrifolia*) (Ubi et al., 2010; Bai et al., 2013), and *PpMADS1* and *PpMADS2* in Chinese white pear 'Suli' (*Pyrus pyrifolia*) (Liu et al., 2012). Many of these genes are up-regulated during the induction of endodormancy and down-regulated during endodormancy release, suggesting that DAMs act as dose-dependent inhibitors of bud break (Yamane et al., 2006; Sasaki et al., 2011). Expression, transgenic, and genetic analyses have all indicated that DAMs are the most likely candidate genes controlling bud dormancy in *Prunus* plants (Yamane et al., 2011). In one study, dehydrin and H^+-ATPase genes were identified as differentially expressed during dormancy transition (Liu et al., 2012). Nevertheless, the entire network responsible for the molecular regulatory mechanism of dormancy in deciduous fruit trees is very complicated and involves many genes. The isolation of a single gene is thus insufficient to elucidate the molecular mechanisms underlying dormancy.

RNA sequencing (RNA-Seq), based on direct sequencing of cDNAs, has been widely applied in transcriptome analyses of dormancy in woody plants. Examples include transcriptome analyses of Japanese apricot buds using 454-pyrosequencing technology (Habu et al., 2012), Chinese white pear 'Suli' (Liu et al., 2012) flower buds, and Japanese pear (Bai et al., 2013). Transcriptome analysis has revealed putative dormancy regulatory pathways involving photoperiod (Olsen, 2010), circadian clocks (Bohlenius et al., 2006), and hormones (Olsen, 2010; Mornya and Cheng, 2011; Dogramaci et al., 2013). Transcriptome profiling by RNA-Seq is more efficient and sensitive than microarray analysis or other techniques (Annadurai et al., 2012), thereby facilitating identification at the molecular level of entire networks regulating dormancy in woody plants.

To better understand the molecular mechanisms of flower bud dormancy in Chinese cherry, we used RNA-Seq technology to sequence a transcriptome library generated from flower buds of Chinese cherry cultivar 'Duanbing'. We obtained high-quality reads and assembled unigenes that provide valuable resources for the identification of genes involved in bud dormancy. We also constructed three gene expression libraries using an upgraded RNA-seq system. By comparing expression patterns of genes in different dormancy states, we identified various pathways and a large quantity of DEGs. Several DEGs involved in phytohormone metabolism and stress response during whole dormancy transition were selected and analyzed by qRT-PCR. Our research provides valuable genetic information for the elucidation of dormancy maintenance and release in flower buds of Chinese cherry.

2. Materials and methods

2.1. Plant materials

Mature, approximately 10-year-old trees of Chinese cherry cultivar 'Duanbing' grown at the fruit tree experimental station of Zhejiang Normal University (Jinhua, China) were used for all experiments. Trees were not pruned or chemically treated during the experimental period. Flower buds were harvested on the following dates: Nov. 14, 21, and 28 and Dec. 5, 12, 19, and 26 in 2012 and Jan. 2 and 9 in 2013. The samples were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Accumulated chilling units (C.U.) were calculated according to the Utah model.

To determine the optimal period of endodormancy release for detection of associated gene expression, we measure the percentage of bud break using the following method. From the current season's growth, 40 shoots of about 30-cm length and with 15–20 buds were collected and placed in water in a 500-ml beaker kept at day/night temperatures of $25 \pm 1/18 \pm 1$ °C, illumination with white light (320 μmol m^{-2} s^{-1} photon flux density) under a 18/6-h photoperiod, and 75% humidity. Every 3 days, the water in the beaker was changed and the basal ends of the shoots were cut. After 25 days, dormancy status was evaluated according to the percentage of bud break. The beginning of bud break was defined as green leaf tips enclosing visible flowers. Flower buds of shoots with bud break percentages ≥50% were considered to be in the critical period of dormancy breaking.

2.2. RNA extraction, library construction, and RNA-seq

After removing three terminal buds, following 4–5 flower buds were collected for RNA extraction. Flower buds were sampled from three biological replicates at each dormancy stage and used to produce an independent pool. Total RNA was extracted from flower buds according to the methods described in Tong et al. (2012). RNA quality was evaluated on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with RNA integrity numbers >7.5 were used for RNA-seq. A pooled transcriptome assembly library was generated by mixing equal quantities of RNA from nine dormant stages.

To create three gene expression libraries consisting of separate RNA extracts from buds at three different dormancy stages, i.e., Nov. 14, Dec. 12, and Jan. 9, equal quantities of RNA from three biological replicates were pooled for each stage. cDNAs used for RNA-seq were synthesized following the protocols of the Beijing Genome Institute (BGI; Shenzhen, China). Poly-(A) mRNA was isolated from total RNA using Oligo-(dT) magnetic beads, and then fragmented in fragmentation buffer. First-strand cDNA was synthesized using short fragments as templates and random hexamer primers. Second-strand cDNA was synthesized using buffer, dNTPs, RNaseH, and DNA polymerase I. Short double-stranded cDNA fragments were purified with a QiaQuick PCR extraction kit (Qiagen, Venlo, Netherlands), resolved with EB buffer for end repair and poly-(A) addition, and ligated to sequencing adapters. Suitable enriched fragments were sequenced using a HiSeq 2000 instrument (Illumina, San Diego, CA, USA). The cDNAs used for qRT-PCR were synthesized from total RNA with a PrimeScript Double Strand cDNA synthesis kit (Takara, Dalian, China) following the manufacturer's instructions.

2.3. De novo assembly and functional annotation

Raw reads obtained from HiSeq-2000 sequencing were filtered to exclude reads containing adaptors, reads with more than 5% unknown nucleotides, and low-quality reads with more than 20% of bases with a quality value ≤10. After assembly of clean reads with Trinity (Gubler, 2011) and removal of redundant sequences using TGICL (Pertea et al., 2003), clusters (prefixed with CL) and singletons (prefixed with Unigene) were finally obtained. Unigenes were aligned by BLASTx (E-value $<1 \times 10^{-5}$) against non-redundant (nr), Swiss-Prot, KEGG, and GO databases. The best-aligning results were used to determine unigene sequence directions. SETScan was used to decide the direction of sequences that could not be aligned to any of the databases. GO annotation was carried out using Blast2GO software (v.2.5.0) (Conesa et al., 2005; Aparicio et al., 2006). KEGG pathway annotation was performed using Blastall software against the KEGG database (Kanehisa et al., 2008). After obtaining a GO annotation for every unigene, WEGO was used to classify GO functions of all unigenes and to understand the distribution of gene functions of the species (Ye et al., 2006). The sequences

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