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Research article

De novo transcriptome analysis in *Dendrobium* and identification of critical genes associated with flowering

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ABSTRACT

Artificial control of flowering time is pivotal for the ornamental value of orchids including the genus *Dendrobium*. Although various flowering pathways have been revealed in model plants, little information is available on the genetic regualtion of flowering in *Dendrobium*. To identify the critical genes associated with flowering, transcriptomes from four organs (leaf, root, stem and flower) of *D. officinale* were analyzed in our study. In total, 2645 flower-specific transcripts were identified. Functional annotation and classification suggested that several metabolic pathways, including four sugar-related pathways and two fatty acid-related pathways, were enriched. A total of 24 flowering-related transcripts were identified in *D. officinale* according to the similarities to their homologous genes from *Arabidopsis*, suggesting that most classical flowering pathways existed in *D. officinale*. Furthermore, phylogenetic analysis suggested that the *FLOWERING LOCUS T* homologs in orchids are highly conserved during evolution process. In addition, expression changes in nine randomly-selected critical flowering-related transcripts between the vegetative stage and reproductive stage were quantified by qRT-PCR analysis. Our study provided a number of candidate genes and sequence resources for investigating the mechanisms underlying the flowering process of the *Dendrobium* genus.

1. Introduction

Flowering is an intricate biological process regulated by a large number of genes (Fitter and Fitter, 2002). Genetic studies have identified five flowering pathways, namely the photoperiod pathway, the autonomous pathway, the vernalization pathway, the gibberellin pathway and the sucrose pathway (Hong and Jackson, 2015; Mouradov et al., 2002). A comprehensive network, with a number of floral integrators, has been established to respond to endogenous or environmental cues. Subsequently, floral integrators, such as *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *AGAMOUS-LIKE* (*AGL*) family genes, and floral meristem identifying genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*), have been identified to be involved in flowering (King et al., 2006; Wang et al., 2008; Zhang et al., 2008). However, knowledge about the molecular genetics of flowering mainly comes from model plants (Wils and Kaufmann, 2017). It is still poorly understood that which key genetic integrators and pathways participate in the flowering process of orchids.

Commercial-scale production of the family Orchidaceae plants, one of the most diverse plant families, has become an economically-important business in the floriculture industry. Orchids, consisting of more than 25,000 species, are highly adapted to diverse natural environments, such as rainforest, grassland and mangrove swamp. *Dendrobium*, the third largest genus in the Orchidaceae family, with over 1450 species, is valued for its use in ornamental gardening and herbal medicine (Adams, 2011; Lu et al., 2012). With market expansion, flowering control becomes very important. However, the underlying regulatory mechanisms of *Dendrobium nobile* flowering remain largely unknown.

A large number of candidate genes associated with floral development and flowering time have been identified in various orchid species. For example, four *DEFICIENS* (*DEF*)*-like MADS-box* genes with distinct floral morphogenetic roles have been identified in *Phalaenopsis* orchids (Tsai et al., 2004); an *APETALA3* (*AP3*)*-like MADS-box* gene associated with floral organ formation and

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initiation has been identified in *Oncidium* 'Gower Ramsey' (Hsu and Yang, 2002); an important role of *SEPALLATA* (*SPL*)-*like* family genes in floral organ formation have also been revealed in *Phalaenopsis* orchids (Pan et al., 2014); *MADS6*, a *GLOBOSA/PISTILLATA-like* gene involved in petaloid formation, has been isolated and characterized in *Phalaenopsis equestris* (Tsai et al., 2005); in *Dendrocalamus latiflorus*, many putative *SPL* family homologs expressed in developing flowers, indicating various roles of *SPL* family genes during flower development (Zhang et al., 2012); and over-expression of *DnAGL19*, a *SOC1/TM3-like* ortholog in *D. nobile*, regulates the expression of *HOS1-FT* genes in *Arabidopsis* (Liu et al., 2016).

Various RNA-sequencing studies identified considerable numbers of *Dendrobium* sequences, promoting the discovery of novel genes involved in flowering process (Meng et al., 2016; Shen et al., 2017). A collection of 15,017 expressed sequence tags from the vernalized axillary buds of *D. nobile* suggested that the networks regulating vernalization-induced floral transition are conserved among plant species (Liang et al., 2012). Another transcriptomic analysis revealed a cytokinin-gibberellin (GA) signal network underlying vernalization, indicating a new insight into the molecular mechanism of floral initiation in *Dendrobium* (Wen et al., 2017). In order to identify potential genes that regulate the floral development and flowering of *D. officinale*, four transcriptomic data sets of *D. officinale* were downloaded from a public database (NCBI SRA). Our data may provide valuable resources to enhance the study on the flowering process of orchids.

2. Materials and methods

2.1. Plant materials and total RNA isolation

D. officinale Kimura *et* Migo seedlings were grown in small pots (3 cm × 3 cm) in a greenhouse at Hangzhou, China, and grown at a temperature of 25 ± 1 °C with a light/dark cycle of 12/12 h and 60%–70% relative humidity. Leaves, stems and roots from 6-month-old seedings and flowers from three-year-old plants at flowering stage were collected. Total RNAs were extracted using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. RNAs were treated with RNase-free DNase I (TakaRa, Dalian, China) to remove genomic DNA. The final RNAs were quantified using Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA) with RIN number > 7.0. For cDNA conversion, the mRNA were fragmented into small fragments using divalent cations under high temperature. The fragmented RNAs were reverse-transcribed to produce the final cDNA library by mRNA-seq sample preparation kit (Illumina, San Diego, USA).

2.2. Raw data and de novo assenbly

Raw transcriptome data from four different organs, including leaves, stems, roots and flowers, of *D. officinale* plants were downloaded from NCBI SRA under accession IDs SRR2014227 (root_repeat 1), SRR2014230 (root_repeat 2), SRR2014236 (stem_repeat 1), SRR2014246 (stem_repeat 2), SRR2014297 (leaf_repeat 1), SRR2014325 (leaf_repeat 2), SRR2014396 (flower_repeat 1) and SRR2014476 (flower_repeat 2) (Meng et al., 2016; Shen et al., 2017). Transcriptome *de novo* assembly was carried out using Trinity program with default parameters (Grabherr et al., 2011). Clean reads were mapped back onto the corresponding contigs, which were large fragments assembled by clean reads. Then, all contigs were assembled to produce unigenes with no extension on either end.

2.3. Functional annotation and identification of flowering-associated genes

All unigenes were searched against several public available databases, including NR, COG, Kyoto Encyclopedia of Genes and Genomes (KEGG), Nt and Swiss-Prot, using the BLASTX algorithm with an *E*-value < 0.00001. For unigene annotation, the Gene Ontology (GO) annotation of unigenes was produced by the Blast2GO program, and the protein products were categorized according to their biological functions by BLAST algorithm-based searching against KEGG database. Flower-specific transcripts were classified into several functional categories by searching against Karyotic Orthologous Groups (KOG) database. The amino acid sequences of flowering-associated genes were downloaded from NCBI database, according to the published flowering network in *Arabidopsis* (Fornara et al., 2010). The assembled transcript sequences from *D. officinale* were subjected to a locally-installed BLASTx progarm for similarity searching against the *A. thaliana* gene sequences at *E*-value < 1.0E-05.

2.4. Identification of flower-specific expressed transcripts

The expression level of each transcript was calculated with the formula described previously (Mortazavi et al., 2008). In brief, the number of mapped reads for each transcript was normalized into a reads per kb per million reads value (RPKM) to calculate transcript expression. Differential expression of each transcript between the two sample groups was calculated using the edgeR package. A transcript is considered to be a flower-specific expressed transcript, if its expression level satisfies the following criteria: (1) the expression of the transcript in flowers should be five times or more than that in roots, stems and leaves. (2) The *p*-value of *t*-test should be smaller than 0.05. (3) The expression of the transcript in flowers should be 1 RPKM or higher. All flower differentially expressed transcripts were analyzed by a K-means algorithm using Multi-Experiment Viewer (MeV) (version 4.9.0) basing on their $\log_2$ values of transcript abundances.

2.5. Phylogenetic tree building

Phylogenetic analysis was carried out using the MEGA 6.0 software (http://www.megasoftware.net/) with a Neighbor-Joining algorithm. The peptide sequences of FT homologs used for phylogenetic tree construction were retrieved from NCBI database (https://www.ncbi.nlm.nih.gov/) (for non-Orchids) and Orchidstra database (http://orchidstra2.abrc.sinica.edu.tw/orchidstra2/) (for Orchids), respectively. The peptide sequences of these FT homologs were listed in Table S1.

2.6. Quantitative real-time RT-PCR (qRT-PCR) validation

Total RNA from different samples was extracted using a Plant RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The *DnActin* (comp205612_c0) gene was set as an internal standard to calculate relative fold differences based on the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method. The primer sequences were designed by Primer premier 6.0 and listed in Table S2. The procedure of qRT-PCR was described as follows: 1 μL of a 1/10 dilution of cDNA in ddH_2O was add to 5 μL of 2 × SYBR Green buffer (Takara, Dalian, China), 0.1 μM of each primer and ddH_2O was then added to a final volume of 10 μL. The PCR conditions were 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 60 s. A histogram was constructed using the average values to visualize the expression levels in different samples. Differences between values were calculated using one-way ANOVA with