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De novo transcriptome assembly and comparative analysis between male and benzyladenine-induced female inflorescence buds of *Plukenetia volubilis*

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ABSTRACT

Plukenetia volubilis is a promising oilseed crop due to its seeds being rich in unsaturated fatty acids, especially alpha-linolenic acid. *P. volubilis* is monoecious, with separate male and female flowers on the same inflorescence. We previously reported that male flowers were converted to female flowers by exogenous cytokinin (6-benzyladenine, 6-BA) treatment in *P. volubilis*. To identify candidate genes associated with floral sex differentiation of *P. volubilis*, we performed *de novo* transcriptome assembly and comparative analysis on control male inflorescence buds (MIB) and female inflorescence buds (FIB) induced by 6-BA using Illumina sequencing technology. A total of 57,664 unigenes with an average length of 979 bp were assembled from 104.1 million clean reads, and 45,235 (78.45%) unigenes were successfully annotated in the public databases. Notably, Gene Ontology analyses revealed that 4193 and 3880 unigenes were enriched in the categories of reproduction and reproductive processes, respectively. Differential expression analysis identified 1385 differentially expressed unigenes between MIB and FIB, of which six unigenes related to cytokinin and auxin signaling pathways and 16 important transcription factor (TF) genes including MADS-box family members were identified. In particular, several unigenes encoding important TFs, such as homologs of CRABS CLAW, RADIALIS-like 1, RADIALIS-like 2, HECATE 2, WUSCHEL-related homeobox 9, and SUPERMAN, were expressed at higher levels in FIB than in MIB. The expression patterns of the 36 selected unigenes revealed by transcriptome analysis were successfully validated by quantitative real-time PCR. This study not only provides comprehensive gene expression profiles of *P. volubilis* inflorescence buds, but also lays the foundation for research on the molecular mechanism of floral sex determination in *P. volubilis* and other monoecious plants.

1. Introduction

Floral sex differentiation plays an important role in fruit set and seed production, and this biological process is regulated by both genetic factors and the external and internal environmental conditions (Tanurdzic and Banks, 2004). Monoecious species provide a comprehensive system for studying the developmental programs underlying the establishment of male and female organs in unisexual flowers (Rocheta et al., 2014). Many studies have reported floral sex determination in monoecious species, such as cucumber (*Cucumis sativus*), bitter gourd (*Momordica charantia*), maize (*Zea mays*), *Jatropha curcas*, and *Quercus suber* (Bai and Xu, 2013; Chen et al., 2016; Rocheta et al., 2014; Shukla et al., 2015; Thompson, 2014; Wu et al., 2010). However, molecular data resources are limited for most monoecious non-model species, especially those without a reference genome, and the mechanisms that control floral sex in different plant species are far from conclusive.

Plukenetia volubilis, a species of the Euphorbiaceae family, also known as Sacha inchi, or Inca peanut, is a perennial oilseed vine and native to the rainforests of South America. *P. volubilis* seeds contain 25–27% protein and 41–54% oil, which comprises approximately 90% unsaturated fatty acids (oleic, linoleic, linolenic) and is rich in vitamins E and A (Chirinos et al., 2013; Gutierrez et al., 2011; Niu et al., 2014). *P. volubilis* oil has great potential economic value in cosmetic, pharmaceutical, and food industries (Chirinos et al., 2013; Hanssen and Schmitz-Huebsch, 2011). Wang et al. (2012) reported the transcriptome analysis of Sacha inchi seeds at the initial and fast oil accumulation stages, and identified 397 unigenes associated with the biosynthesis of fatty acids. Recently, an *in vitro* regeneration system for *P. volubilis* has been successfully established using cotyledons as explants (Dong et al., 2016). These studies lay the foundation for further gene function studies and utilization of *P. volubilis*. However, studies on flower

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development, especially floral sex determination, in *P. volubilis* remain limited. *P. volubilis* is monoecious, with separate female and male flowers on the same inflorescence. Only one or two female flowers are at the basal-most node, and numerous male flowers are radially set around the narrow raceme-like inflorescence axis (Gillespie, 1993). We previously developed an efficient method to convert most of the male flower buds to female flower buds by exogenous 6-benzyladenine (6-BA) treatment (Fu et al., 2014). Therefore, the molecular mechanisms of floral sex determination in *P. volubilis* are worthy of further research.

Many studies have shown that phytohormones are significantly involved in modulating floral sex-developmental processes in a variety of species. In general, ethylene, cytokinin, and auxin promote female sex development, while gibberellic acid promotes male sex development (Chailakhyan and Khryanin, 1978; Heslopharrison, 1956; Orozco-Arroyo et al., 2012; Pan et al., 2014; Yin and Quinn, 1995). Some phytohormone biosynthesis and perception genes that affect sex determination have been discovered, such as the key regulatory genes of ethylene biosynthesis, the 1-aminocyclopropane-1-carboxylic acid synthase (ACS) genes *CmACS-7* and *CmACS-11*, which regulate the sex determination in melon (Boualem et al., 2008). Some transcription factors (TFs) play important roles in regulating floral sex development, such as SUPERMAN (SUP) (Kazama et al., 2009), MYB (Song et al., 2013; Wu et al., 2010), and basic helix-loop-helix (bHLH) (Gremski et al., 2007), in different plant species. In addition, MADS-box TF genes are also implicated in sex differentiation of male and female flowers in unisexual plants. Chawla et al. (2015) demonstrated that *HrAP1* showed female-specific expression, while *HrAP2* was expressed particularly in male flowers of seabuckthorn (*Hippophae rhamnoides*). *SpAP*3 of spinach (*Spinacia oleracea*) is strongly expressed in male flowers and weakly expressed in female flowers, and *SpPI* is expressed early in male flower development (Pfent et al., 2005). These results indicate that floral sex determination is governed by many signals and complex transcriptional regulatory networks.

In recent years, transcriptome-sequencing technology has become increasingly common for unravelling the genetic networks that regulate floral sex determination and development in many species. Rocheta et al. (2014) analyzed the transcriptome of male and female flowers in monoecious *Quercus suber* using 454 pyrosequencing technology, and identified some genes involved in pollen development and ovule formation. In the transcriptome analysis of flower buds at different developmental stages in *Jatropha curcas*, 15 sex-related genes contributing to stamen differentiation and embryo sac development were obtained (Xu et al., 2016). Other studies have also been conducted to detect the candidate genes underlying sex determination in a variety of plant species, such as wild grapevine (*Vitis vinifera*) (Ramos et al., 2014, 2017), bitter gourd (Shukla et al., 2015), and castor bean (*Ricinus communis*) (Tan et al., 2016). Although many genes involved in regulating floral sex have been identified in different species, the gene expression information in florescence buds and the factors regulating floral sex in *P. volubilis* remain poorly understood.

To reveal sex differences in *P. volubilis* at the transcription level, we performed *de novo* transcriptome assembly and comparative analysis on control male inflorescence buds (MIB) and female inflorescence buds (FIB) induced by 6-BA. More than 104.1 million clean reads and 57,664 unigenes were obtained from the two transcriptome libraries. We identified multiple phytohormone-related genes and some important transcription factors (TFs) genes including MADS-box family members, some of which might play important roles in flower development and floral sex determination in *P. volubilis*.

2. Materials and methods

2.1. Plant material

Two-year-old *P. volubilis* trees were grown at Xishuangbanna Tropical Botanical Garden (21°54′ N, 101°46′ E, 580 m asl), Chinese Academy of Sciences, located in Mengla County, Yunnan Province, China, under natural conditions. Because only one to two female flowers are located near the base of the inflorescences, it is difficult to obtain female flower buds in the early developmental stage of inflorescence. To obtain female flower buds, *P. volubilis* trees were treated with 20 mg/L 6-BA in July–August 2014, as previously reported (Fu et al., 2014); control trees were sprayed with distilled water containing 0.05% (v/v) Tween-20. When the first poly-female inflorescence (> 2 female flowers) was observed, the fourth to sixth inflorescences of the branch were selected as FIB. The selected inflorescences were less than 0.5 cm in length. MIB were selected from control inflorescences during a similar developmental stage. Each sample consisted of more than 20 male or female inflorescence buds, respectively. The collected samples were immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

2.2. RNA isolation, library construction, and transcriptome sequencing

Total RNA of each sample was isolated using Trizol reagent (Invitrogen, USA) and purified using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' protocols. The quality and quantity of total RNA were assessed using a 1.0% agarose gel and a Nano-Drop ND1000 spectrophotometer, and RNA integrity was evaluated using an Agilent 2100 Bioanalyzer. Then, the isolated RNA samples were prepared for construction of transcriptome libraries and quantitative real-time PCR (qRT-PCR) validation.

Poly (A) mRNA was isolated and enriched from total RNA using oligo (dT)-attached magnetic beads according to the manufacturer's (Illumina) instructions and then chemically broken into short fragments in fragmentation buffer. Using these short mRNA fragments as templates, random hexamers were used as primers to synthesize first-strand cDNA. Subsequently, second-strand cDNA was synthesized in a buffer containing dNTPs, DNA polymerase I, and RNaseH. Short cDNA fragments were purified with a QIAquick PCR extraction kit and resolved in extraction buffer (10 mmol/L Tris-HCl, pH 8.4). After the purified cDNA ends were repaired and single nucleotide adenine (A) was added, the fragments were ligated to sequencing adapters. The suitable fragments were screened and purified as templates for PCR amplification to enrich the cDNA. The cDNA libraries were sequenced using an Illumina HiSeq™ 2000 sequencing platform at the Beijing Genomics Institute (BGI, Shenzhen, China), and the raw reads were generated in a 90-bp paired-end format.

2.3. De novo transcriptome assembly and unigene functional annotation

Before data analysis, the raw reads were filtered using the Filter_fq (an internal program of BGI) by removing adaptor reads, low-quality reads (containing more than 20% bases with Q-value ≤ 10), and reads containing more than 5% unknown nucleotides. The clean reads obtained from each library were assembled separately, and pooled reads of both libraries were also assembled *de novo* into unigenes using Trinity program (Grabherr et al., 2011). The longest non-redundant unigenes were acquired by removing sequence splicing and redundancy using TGI Clustering tools (TGICL) (Pertea et al., 2003). Then the unigenes were divided into two classes: "clusters", with the prefix "CL", and "singletons", with the prefix "unigene". The transcriptome data have been deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) database under the accession number SRP101414 (ftp://ftp-trace.ncbi.nlm.nih.gov/sra/review/SRP101414_20171019_083700_a3f2a910685f5b07f5f45a5fc1fdb389).

For further annotation of unigenes, all the assembly unigenes were first searched against the NCBI non-redundant protein database (Nr) and Swiss-Prot protein database using BLASTx alignment (E-value < 1.0E-05), and the NCBI non-redundant nucleotide sequence (Nt) database using BLASTn (E-value < 1.0E-05). The best aligning