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Differential expression profiles and pathways of genes in drought resistant tree species *Prunus mahaleb* roots and leaves in response to drought stress

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ABSTRACT

Prunus mahaleb exhibits tolerance to drought as woody plant genotype, shows great potential for cherry rootstock breeding, while the molecular mechanism for its respond to water deficiency is still not fully understood. The physiological and RNA-seq approaches were used to investigate the transcriptome mechanisms that allow *P. mahaleb* to survive in arid environments. 1, 573 differentially expressed genes (DEGs) involved in drought response were selected from well-watered and drought-stressed *P. mahaleb* leaves (517) and roots (1, 056). Carbohydrates metabolism, hormone signal transduction and secondary metabolites were intensive pathways involved. Glycogen biosynthesis and glycosyl hydrolases genes were reduced to rebuild energy homeostasis, *NCED* homology genes were induced for stomatal regulation and water conservation. The expression of linolenic acid and amino acids synthesis related genes was generally increased to enhance drought tolerance. CBF/NF-Ys, MYB, WRKY and U-box as TFs may regulate key functional genes to adapt the stress. It is indicated differences pertaining to the molecular mechanisms occurring in tree roots vs. shoots in response to drought stress. A core set of 32 candidate genes were identified that could function as targets for detailed functional studies of drought responses at molecular level in *prunus* family. Furthermore, these genes may be used to potentially breed trees with drought tolerance.

1. Introduction

Drought is a major abiotic stress for plant survival and severely impacts on the yield and quality in agricultural production systems (Chaves et al., 2003; Sivritepei et al., 2008; Küçükyumuk et al., 2015). Given the increasing threatens of water deficiency globally, it is of great importance for breeders to understand the molecular responses of plants to drought stress and develop novel molecular approaches to enhance the drought tolerance of plants. The natural germplasm resources with excellent drought resistance are ideal materials to reveal the mechanism on plant responses and survival under water deficient conditions.

Prunus are the most economically beneficial population for producing fruits, such as peach, cherry, plum and apricot. Most of them are sensitive to water deficits, while *prunus mahaleb* is one of the drought resistant species shows great potential for cherry rootstock breeding (Hrotkó, 2016). *P. mahaleb* is native to Europe and Western-Asia (Rehder, 1927; Hrotkó, 2016). It grows in thickets and open woodland on dry slopes, especially in central and southern Europe typically in thickets on dry karst areas (Faust and Surányi, 2010). It was reported that this species can survive in extremely drought conditions, the drought resistance of this species is due to its deep root systems allowing more water uptake, thus maintaining a stable hydraulic conductivity (k) in stem throughout the drought season (Nardini et al., 2015).

Various strategies of avoidance and tolerance are taken in plants to adapt to drought stress. The integrated responses involve changes at whole plant level, such as shoot-root carbon allocation, plant growth rate, leaf and root morphology, leaf abscission, stomatal conductance and photosynthesis (Sivritepe et al., 2008; Chaves et al., 2009; Pucholt et al., 2015). Besides that, molecular changes induced by drought stress were also examined by remodelling of the transcriptome, including upregulation of stress signalling, energy metabolism, secondary metabolites production, transcription factors and defence processes (Singh et al., 2013; Dietze et al., 2014). Apparently, the responses of woody plants coping with extreme drought are involved the morphological, physiological, biochemical and molecular processes. Jiménez et al. (2013) stated that drought resistance of *prunus* plants is closely related

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to its genetic background. Thus, besides the physiological and morphological explanation, it is necessary to explore the unique molecular and biochemical mechanisms associated with drought tolerance in some extreme plants. *P. mahaleb* grows well in water-limited areas with native drought resistance and plays an important role in economic and ecological environment. However, few studies have been reported to reveal the molecular mechanisms of drought tolerance in this species.

RNA-seq has been used successfully to identify abiotic stress related genes, implicating several biosynthetic pathways that assist in the overall tolerance to drought stress. The molecular responses of plants to drought has been well documented in model plant *Arabidopsis* (Sakuraba et al., 2015), rice (Oono et al., 2014), Morus L. (Wang et al., 2014), B. juncea (Bhardwaj et al., 2015), and barley (Hübner et al., 2015) using high-throughput transcriptomics. For *Prunus* plants, Wang et al. (2015) characterized the transcriptome of *P. mongolica* under drought stress, and shed new lights on the molecular mechanisms underlying the response to drought in this species.

Generally, root system initially experiences and signals drought in plants, thus, playing a key role in coping with water stress. This is consistent with the finding that there are genes specifically expressed in certain root types under drought stress (Ghosh and Xu, 2014). *MdNRT2.4* and *MpNPR1-2* were found specifically expressed in *Malus* roots under drought (Bassett et al., 2014). Since drought responses may varied in different plant organs or tissues (Zhou et al., 2007), the analysis in both mature leaves and root tips is critical for plants to adapt to water deficits.

The objectives of this research were to identify stress-induced patterns of gene expression and select candidate genes related to drought resistance under severe drought in *P. mahaleb*. Transcriptome high-throughput sequencing was performed in root and leaf tissues of *P. mahaleb* plant under well-watered and water-deficient conditions. respectively to profile the gene expression, together with physiological process and biochemical functions, to provide a comprehensive analysis of drought acclimation in this species. The acquired genes, pathway analysis and other information may provide new insights into the molecular mechanisms underlying *P. mahaleb* response to water stress.

2. Materials and methods

2.1. The growing conditions, experimental design and sampling

The experiments were conducted at Northwest A & F University, Yangling, China (34°20′N, 108°24′E). Two-year old *P. mahaleb* seedlings were grown in glasshouse with day/night temperature of 28/18 °C. Sixty pots containing potting humus were divided into two equal groups, and one group was use for drought treatment and for the other one for well-water control.

Seedlings were grown one year in field in 2014, and then transplanted into 4.5L pots and placed in glasshouse on 14th February 2015. They were irrigated every 3 days to field capacity as needed and fertilized weekly with Hoagland's solution. All plants were irrigated as the same manner before treated, the soil moisture in pots was maintained about 75% of field capacity. The water-stress treatment was imposed by stopping irrigation, while the control treatment was continued watering. Plant drought stress was monitored based on leaf relative water content (RWC). On day 15, the first time of leaf wilting appearance, and the leaf RWC of water-stressed plants was 65% (vs 89% in control). All plants were sampled, including leaves under water-stressed and control treatments (LS1, LS2, LS3, LCK1, LCK2 and LCK3 respectively) and root tip under water-stressed and control treatments (RS1, RS2, RS3, RCK1, RCK2 and RCK3). Mature and healthy leaves (4–6th leaves from the apical meristem) were sampled from each plant and immediately frozen in liquid nitrogen. Root tip tissues were harvested at the same time after quickly washing off and sucking moisture, and then frozen in liquid nitrogen. The frozen tissues were stored at −70 °C for further analysis.

2.2. Physiological measurements

RWC was calculated using the following equation, RWC(%) = [(FW − DW)/(TW − DW)] × 100. Superoxide dismutase (SOD) activity was determined using NBT-illumination method (Giannoplitis and Ries 1977); peroxidase (POD) activity was determined using guaiacol colorimetric method, and catalase (CAT) activity using hydrogen peroxide method (Zou, 2000). Alondialdehyde (MDA) content was determined by barbituric acid colorimetric method (Zhao et al., 1994a,b). Proline (Pro) content was determined using ninhydrin colouring and spectrophotometry method (Zhang et al., 1990). The leaf samples for physiological measurements were harvested at 4 stages according to pot soil moisture content: 75%–80% (well-watered), 55%–60% (mild drought), 40%–45% (moderate drought) and 30%–35% (severe drought)of field capacity.

2.3. Preparation of total RNA and cDNA for transcriptome sequencing

Total RNA was isolated from plant root tips and leaves by using TRIzol® reagent according to the manufacture's protocol. 0.1 g root tips or leaf tissues were well ground into fine powders and added 1 ml TRIzol® reagent. The extracted liquid was added into 0.2 ml of chloroform and centrifuged at 1200rcf for 15 min at 4 °C, and the cleared supernatant was transferred into a new tube and added into 0.5 ml isopropanol. Then, tubes were incubated at room temperature for 10 min and centrifuged at 1200rcf for 10 min at 4 °C. 1 ml of 75% ethanol was added into the tube to wash the RNA deposit and then dried and suspended in RNase-free water. The RNA quality was controlled using Nanodrop, Qubit 2.0 and Aglient 2100. After that, the RNA was used for cDNA library constructionusing Takara PrimeScript RT reagent Kit.

2.4. Sequencing and de novo assembly of the prunus mahaleb transcriptome

Qubit2.0 and Agilent 2100 were used to test the concentration of the library and insertion fragment size (Insert Size), respectively. The Q – effective concentration of library accurate quantitative PCR method was used to ensure the quality of library. 12 cDNA libraries were sequenced by SBS (Sequencing By Synthesis) sequencing technology with an Illumina HiSeq™ 2500 sequencer. Read length is PE125. *De novo* assembly of the transcriptome was performed by Trinity software. To ensure a uniform transcriptome reference across samples, all clean reads were pooled together for assembly, then clean reads of each sample was individually aligned to the assembled transcriptome reference to get mapped reads for subsequent analysis.

2.5. The identification of differential expressed genes and pathway analysis

In order to define the set of expressed genes, raw read counts were normalized to RPKM (Reads per Kilobase per Million), above or equal to 1 were filtered out. Differential expressed genes (DEGs) were selected by performing the negative binomial test implemented in the DESeq package. In present study, the DEGs were filtered by their fold changes (> 2) and FDR (Benjamini-Hochberg method adjusted p-values < 0.01) as screening standards.

To identify the Differential Expression Genes (DEGs), they were annotated by comparing to previously annotated genes in public databases, NCBI non-redundant (NR) database, Swiss-Protdatabase, Gene Ontology, euKaryotic Orthologous Groups/Clusters of Orthologous Groups (KOG/COG). Pathway analysis was implemented using Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Then, HMMER Software and Pfam Information database were used for unigene sequences prediction of amino acids comments. GO terms and KEGG pathways fulfillig the criterion of a Bonferroni-corrected p-value ≤ 0.05 were defined as significantly enriched in DEGs. BLAST E-value $\leq 10^{-5}$ and HMMER E-value $\leq 10^{-10}$ were set as select