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Research paper

Physiological and transcriptomic analysis revealed the involvement of crucial factors in heat stress response of *Rhododendron hainanense*

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ABSTRACT

Molecular regulatory mechanism of heat stress response (HSR) in *Ericaceae* remains unknown. Here, we sought to identify HSR mechanisms in *Rhododendron hainanense*, a *Ericaceae* species, through a combination of physiological and transcriptomic studies. The levels of MDA, H_2O_2, Pro, SOD, CAT and APX in leaves of *R. hainanense* were analyzed to characterize a dramatic difference in varied temperature treatment. Also, three sequencing libraries, including one control and two heat stress (HS)-treated samples, were constructed for comparative transcriptomic analysis. By Illumina sequencing and Trinity strategy, 350 million clean reads (average length = 149 bp) was assembled into 183,486 unigenes. According to analysis of differential expression genes (DEGs), a total of 2658 DEGs were obtained. Moreover, a complex interaction network of 982 DEGs was established, of which master portions were comprised of 109 transcription factors (TFs). Importantly, integrated differential expression profiling, qRT-PCR and functional analysis, several TFs of *R. hainanense* (ABR1, IAA26, OBF1, LUX, SCL3, DIV, NAC29, NAC72 and TCP3) and their potential regulations for the crosstalk between hormonal signal and HSR were identified. These findings will contribute to our understanding of the regulatory mechanisms of HSR in *R. hainanense*, breeding cultivars with improved thermotolerance.

1. Introduction

Rhododendron hainanense Merr., a member of the family *Ericaceae*, is distributed specifically in the temperate and mountainous districts of Hainan and Guangxi provinces of China. *R. hainanense* is not only an important integrant of forest ecological systems from subtropical to warm temperate zones, but is also an important species in landscaping and bonsai for the construction of urban ecosystems (Shi et al., 2010). Additionally, a variety of triterpenoids and flavonoids have been extracted from *R. hainanense*, indicating a potential economic and medicinal value (Zhao et al., 2012; Zhao, 2013). However, *R. hainanense* favors cold and humid environments, and grows better at ~25 °C, thus heat stress (HS) has become an increasingly major problem for its *ex-situ* conservation and landscape application with the progression of global warming.

Temperatures above the normal optimum result in HS, which has negative effects on cell division and growth, and can lead to severe retardation in plant growth and development (Kotak et al., 2007). As sessile organisms, plants have evolved a series of HS response (HSR) mechanisms to adapt to the changing environments, but the complexity of the HSR network remains largely unclear (Larkindale et al., 2005b; Kotak et al., 2007; Ohama et al., 2017). With the development of molecular techniques, such as next-generation sequencing for RNA sequences (RNA-seq) and chromatin immunoprecipitation sequencing, many HS-inducible genes have been identified in the plant HSR network (Gonzálezschain et al., 2015). Among these genes, transcription factors (TFs) have critical roles in the conversion of stress signal perception and the regulation of transcriptional network in the HSR

Abbreviations: AGO, Argonaute; AMY3, alpha-amylase-like 3; APX, ascorbate peroxidase; ARF5/MP, Auxin response factor 5; BAM1, beta-amylase 1; CAT, catalase; CK, control check; COG, Clusters of Orthologous Groups of proteins; DEG, differential expression gene; ERF1, Ethylene response factor 1; EXPA10, expansin A10; FPKM, Fragment Per Kilobase of exon model per Million mapped reads; GO, Gene Ontology; H_2O_2, hydrogen peroxide; H-HS, high-heat stress; HS, heat stress; HSR, heat stress response; IAA26, Indole-3-acetic acid inducible 26; KEGG, Kyoto Encyclopedia of Genes and Genomes; MDA, malondialdehyde; M-HS, middle-heat stress; NR, NCBI non-redundant protein sequences; Nt, NCBI non-redundant nucleotide sequences; Pfam, protein family; PRF, phytohormone-responsive factor; Pro, proline; PTGS, post-transcriptional gene silencing; RDR6, RNA-dependent RNA polymerase 6; RNA-seq, RNA sequences; SDE3, Silencing defective protein 3; SOD, superoxide dismutase; TF, transcription factor; TPL, TOPLESS; XRN4, EXORIBONUCLEASE 4

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(Mishra et al., 2002; Sato et al., 2014; Gonzálezschain et al., 2015; Guo and Qin, 2016; Ohama et al., 2017). Therefore, to enhance thermotolerance in plants, TFs are indispensable as master regulators for activating signaling cascades. To date, various TF families such as AP2/EREBP, bZip, NAC, WRKY, MYB and bHLH have been confirmed to be involved in the intricately regulatory network of the HSR in model plants and crops (Larkindale et al., 2005a; Larkindale et al., 2005b; Kotak et al., 2007; Merret et al., 2013; Gawroński et al., 2014; Sato et al., 2014; Guo and Qin, 2016). However, the responsibility of key TFs for the HSR in *Ericaceae*, especially in *R. hainanense*, is poorly understood.

In this study, we first determined the typically physiological indexes in leaves of *R. hainanense* under various temperature treatments, and identified these *R. hainanense* samples suitable for comparative transcriptomic analysis. The transcriptomic sequencing of *R. hainanense* leaves was performed using Illumina technology, and the assembled unigenes were functionally annotated. Subsequently, the HS-responsive genes (*i.e.*, differentially expressed genes; DEGs) were screened by the DESeq method, and their interaction network was constructed. Among the DEGs, TFs were further screened and identified. Based on the expression and function analysis, we identified several key TFs and their corresponding regulatory roles in crosstalk between hormonal signaling and HSR in *R. hainanense*, which will contribute to improving our understanding of the HSR in plants.

2. Materials and methods

2.1. High heat stress treatment and collection of leaves

R. hainanense plants that had grown for two years at Hainan University (20.1N, 110.3E, 6 m above sea level) were chosen as the experimental material. *R. hainanense* were planted in artificial climate chambers and treated under a range of temperatures (day/night, 14 h/10 h, 2000 lx, 70–80% RH): 25/22 °C (control), 35/28 °C, 40/32 °C, for 6 days. All the plants were divided into 25 °C group (CK), 35 °C group (middle-heat stress, M-HS) and 40 °C group (high-heat stress, H-HS), and each group included three replications. During the treatment, the plants were watered as needed. The leaves of plants in each group were collected as samples with three replicates per group. Some of the leaves were immediately frozen in liquid nitrogen and stored at −80 °C until use for RNA extraction. The other samples were used for the physiological and biochemical change determinations. For each group, the contents of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), proline (Pro), catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) were determined using the respective Solarbio detection kits. The experimental data were analyzed by SPSS software.

2.2. cDNA library preparation and sequence data analysis and assembly

Total RNA was extracted from samples using RNeasy Plant Mini Kits (Qiagen, Inc., Valencia, CA, USA). RNA degradation and contamination were monitored on 1% agarose gels. RNA purity was assessed using a NanoPhotometer spectrophotometer (IMPLEN, CA, USA). The RNA concentration was measured using the Qubit RNA Assay Kit with a Qubit 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit for the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total of 1.5 μg of RNA per sample was used as the input material for the RNA sample preparation. Sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was performed using divalent cations under an elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5×). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H^-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. The remaining overhangs were converted into blunt ends *via* exonuclease/polymerase activity. After adenylation of the 3′ ends of the DNA fragments, NEBNext Adaptor with hairpin loop structure was ligated to prepare for hybridization. To select cDNA fragments preferentially 150–200 bp in length, the library fragments were purified with an AMPure XP system (Beckman Coulter, Beverly, USA). Then, 3 μL of USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min, followed by 5 min at 95 °C before PCR. Then, PCR was performed with Phusion High-Fidelity DNA polymerase, universal PCR primers, and Index (X) Primer. Finally, the PCR products were purified with the AMPure XP system and the library quality was assessed using the Agilent Bioanalyzer 2100 system.

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and paired-end reads were generated. Raw data in the fastq format were first processed through in-house perl scripts. In this step, clean data were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data by Illumina Casava 1.8. At the same time, Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. All the downstream analyses were based on clean data with high quality. Transcriptome assembly was accomplished using Trinity software (Grabherr et al., 2011) with min_kmer_cov set to 2 as the default and all other parameters set to the default. All transcriptomic data can be accessed in BioProject PRJNA389341.

2.3. Unigene annotation

To understand their functions, the *R. hainanense* unigenes were annotated against the following public databases, TAIR10, Nr (NCBI non-redundant protein sequences) and Nt (NCBI non-redundant nucleotide sequences), Swiss-Prot and COG (Clusters of Orthologous Groups of proteins), by NCBI blast 2.2.28$^+$ with an E-value cut-off of 10^{-5}. In most cases, unigenes of *R. hainanense* could achieve comparable annotated results in different public databases owing to the conserved regions of genes that determine the main function. If the query hit to the different subjects in different databases, considering extensive studies for *Arabidopsis* genes, we assigned *Arabidopsis* genes to the unigene sets at first, followed by Swiss-Prot, Nr and Nt. Gene Ontology (GO) functional classifications and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway assignments were performed by Blast2GO v2.5 (Götz et al., 2008) and KOBAS software (Kanehisa et al., 2008), respectively.

2.4. Differential expression and interaction analysis

The expressed levels of genes were estimated using Fragment Per Kilobase of exon model per Million mapped reads (FPKM) (Trapnell et al., 2010) by RSEM software (Dewey and Li, 2011). The differential expression analysis of two conditions/groups was performed using DESeq (Anders and Huber, 2010). DESeq provides statistical routines to determine differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate (Benjamini and Hochberg, 1995). Genes with an adjusted P-value (Q value) < 0.05 found by DESeq were assigned as DEGs. The clustering analysis of DEGs was performed by hierarchical clustering, H-cluster, and SOM using pheatmap R package, respectively. The clustering result was manually corrected, based on the results of clustering analysis and differential expression analysis, to select DEGs in the same cluster with similar expression patterns. The GO enrichment of DEGs was performed by Blast2GO v2.5 (Götz et al.,