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Transcriptome Analysis of *Cocos nucifera* L. Seedlings Having Contrasting Water-Use Efficiency (WUE) under Water-Deficit Stress: Molecular Insights and Genetic Markers for Drought Tolerance †

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Abstract: Perennials utilize complex adaptive strategies and molecular mechanisms to cope with water-deficit conditions. Hence, to gain molecular insights regarding the water-deficit stress, two-year-old coconut seedlings of the varieties Kalpa Sree and Kalpatharu were subjected to soil water-deficit regimes (25% of available of soil moisture and control). Biochemical, physiological and growth parameters underlying water-deficit stress revealed the differential enzymatic anti-oxidants, lipid peroxidation status and water use efficiency trait between the genotypes investigated. The whole plant water use efficiency at control condition was significantly low in Kalpatharu (4.06) compared to Kalpa Sree (4.74). Nevertheless, under severe stress [25% ASM] Kalpatharu exhibited highest WUE (5.68) as against dwarf variety Kalpa Sree (3.84). Furthermore, the leaf transcriptome profiles of the control and water-deficit stressed seedlings were examined utilizing paired-end RNA-Seq. In total, ~7300 differentially expressed genes have been identified between the seedlings under water-deficit stress and control. Analysis of control and stressed Kalpasree leaf transcriptome showed significant upregulation of PHLOEM PROTEIN 2-LIKE A1-like, WRKY transcription factor 40 isoform X1 and downregulation of glycerol-3-phosphate acyltransferase 3 transcripts. On the other hand, upregulation of transcripts encoding polyamine oxidase, arabinose 5-phosphate isomerase among others and downregulation of aquaporin PIP1-2 transcript was documented in Kalpatharu leaves. Besides, long non-coding RNA and genic SSRs were also identified from the transcriptome data to further enrich the genomic resources of coconut palm which would pave way for its utilization in developing climate-smart coconut crop. The implication of this study in molecular dissection of the adaptive response of coconut to the soil-water deficit is also discussed.

Keywords: coconut seedlings; climate-smart; drought adaptation; genic markers; RNA-seq

1. Introduction

Coconut palm (*Cocos nucifera* L.) is an important plantation crop of humid tropics. Coconut is grown in 13 Mha spread over 90 countries including the Philippines, Indonesia, India, Brazil and Sri Lanka with an estimated production of 69,836.36 million nuts. In India, coconut is grown in an area of 2.088 Mha with a production of 22,167.45 million nuts [1]. Among the various biotic and abiotic stresses that hinder the productivity of coconut, moisture-stress profoundly influences its annual yield. The amount of rainfall and dry spells preceding 4 years [2,3], weather variables [4] also

influence the productivity of coconut. Further, dry spell of about 200 days could further reduce the yields of coconut palms [4]. It has also been documented that coincidence of dry spell with sensitive stages such as inflorescence and nut development greatly influences the yield of palms. Eventhough the assessment of impact of climate change on coconut cultivation emphasis that coconut plantations would benefit owing to increased CO_2 but the caveat is that it requires appropriate quantity of moisture supply. Furthermore, genetic improvement of local tall cultivars adapted for moisture-deficit stress and non-limiting supply of other inputs are also suggested as a long-term measure for managing water stress in coconut. Considering the importance of drought/water stress in coconut plantation sector, ICAR-CPCRI has devised research programs to phenotype coconut varieties and to devise suitable drought mitigation systems and to develop climate smart coconut. To achieve this objective rigorous screening of tall and dwarf genotypes of coconut for water use efficiency (WUE) and to analyze their molecular response are pertinent.

2. Experiments

2.1. Coconut Genotypes

Seedlings of coconut varieties Kalpa Sree and Kalpatharu grown in large plastic buckets (64 × 49 cm) of 100 kg dry soil capacity were investigated for their water use efficiency (WUE), the morphological, physiological, biochemical and molecular changes linked to water deficit stress.

2.2. Drought Experiment

Coconut seedlings of each variety were exposed to moisture regimes: 100% available soil moisture (ASM)-control; and 25% ASM-stressed. Briefly, after overnight saturation of soil, the amount of water that must be provided daily to maintain the 100% ASM (20% moisture content) (https://nrcca.cals.cornell.edu/soil/CA2/CA0212.1-3.php) was estimated. Soil moisture was measured using soil moisture probe (PR2 profile probe, delta T devices, UK) supplemented with a data logger. Every day the soil moisture content was measured and the amount of water required to bring back the soil to 100% ASM was determined and recorded. Stressed seedlings received 25% of the quantum of water as that of control.

2.3. Biochemical Characterisation

Epicuticular wax content was estimated following the method of Ebercon et al. (1977) [5]. Lipid peroxidation was estimated as malondialdehyde (MDA) equivalent following the methodology enumerated by Heath and Packer (1968) [6]. Assays for enzymatic antioxidant were performed [7,8].

2.4. RNA Extraction, Library Preparation and Sequencing

Based on the physiological data, coconut leaf samples subjected to moisture deficit stress and control were collected in the morning (around 8:00–9:00 A.M., IST) and flash frozen in liquid nitrogen. Total RNA was isolated following guanidium thiocynate method and quality of the isolated RNA was assessed on 1% formaldehyde denaturing agarose gel and quantified using Qubit® 2.0 Fluorometer. The libraries were prepared with total RNA (~1 μg) following Illumina TruSeq Stranded mRNA Library Preparation Kit as per the manufacturer's protocol. The amplified libraries were analyzed on Bioanalyzer 2100 (Agilent Technologies) using High Sensitivity (HS) DNA chip as per manufacturer's instructions. The library was loaded into Illumina platform for cluster generation and sequencing.

2.5. Transcriptome Analysis

A master de novo assembly was generated using Trinity v2.1.1 (at default parameters, kmer 25). Transcripts were further processed for unigenes prediction using CD-HIT package v 4.6.1. The unigenes were subjected to similarity search against NCBI's non-redundant (nr) database, Uniprot, KOG, and Pfam databases using the BLASTX algorithm v2.2.30 [e-value threshold of 1×10^{-5}].

Ortholog assignment and mapping of the unigene to the biological pathways were performed using KEGG automatic annotation server (KAAS). Simple sequence repeats (SSRs) were identified in unigene sequences of master assembly using the MISA perl script. For calculating the fold change in gene expression, the ratio of basemean value of treated sample and basemean value of control sample (i.e.,; FC = basemean of Treated/basemean of Control) was considered and subsequently log 2 (FC) and *p*-value was also calculated to infer the significance of genes. Differential gene expression among the genotypes were assessed using DESeq (bioconductor). Plant related lncRNA sequences were obtained from ensemble plant database present in RNAcentral v-14 (https://rnacentral.org) and BLASTN homology search were performed taking 85,516 unigene sequences (having no blast hit against NR database) against RNAcentral database with an e-value threshold of 1×10^{-5}.

3. Results

3.1. Water Use Efficiency (WUE)

The whole plant WUE significantly differed amongst genotypes. At 100% ASM it was significantly low in KT (4.06) compared to Kalpa Sree (4.74). Both the biomass accumulation and water consumption was low in Kalpatharu compared to that of Kalpa Sree. However, under severe water stress (25% ASM) the tall variety kalpatharu had the highest WUE (5.68) as against dwarf variety Kalpa Sree (3.84). This suggested that under water limited condition tall genotypes are more efficient in utilizing the water and are more adaptive as compared to dwarfs (Figure 3).

(**a**) (**b**)

Figure 1. Imposition of water-deficit stress and morpho-agronomic features of seedlings (**a**) Kalpa Sree and (**b**) Kalpathrau).

3.2. Physico-Chemical Parameters

Epicuticular wax (ECW) is known to act as a barrier for the loss of water through transpiration from the plants. Kalpatharu grown under moisture stress (25% ASM) exhibited the highest ECW (88.59 μg cm^2) while the leaves of the Kalpa Sree showed ECW content of 54.63 μg cm^2. Mean value of MDA (Malondialdehyde), which is an indicator of lipid peroxidation, was high in stressed plants grown under 25% ASM (31.8854 mmol g^{-1}fr.wt) and the MDA content decreased with water status of the plants. Among the enzymatic antioxidants, SOD showed an increase in specific acticity from 3.49 to 5.98 units/mg of protein in KT whereas in the leaves of Kalpa Sree seedlings the increase was minimal from 2.35 to 2.51 units/mg of protein. However both the genotypes showed a huge increase in specific activity of polyphenol oxidase (Kalpa Sree from 0.14 to 1.18; Kalpatharu 0.16 to 1.37) though kalpatharu show relativly a high increase in PPO activity

3.3. Transcriptomic Features of Drought Tolerance

Four cDNA libraries yielded approximately 158.96 million bp reads with an average transcript length of 829 bp and transcript N50 value of 1225. Analysis of transcrpt length distribution revealed that a maximum of 68,482 transcripts belonged to class 300–400 bp. Transcripts were further

processed for unigene prediction using CD-HIT package v 4.6.1 which predicted a total of 162,483 unigenes with a mean unigene length of 786 bp. A maximum of 60,676 unigenes predicted from the transcripts belonged to length class 300–400 bp. The master unigene analyzed for functional annotation and expectedly the top-hit species distribution revealed that majority of the hits were found to be against the related species Elaeis guineensis followed by Phoenix dactylifera. EuKaryotic Orthologous Groups, or KOGs analysis showed that most enriched KOG categories for unigenes were (a) signal transduction mechanisms (T) followed by (b) general function prediction only and c) post-translational modification, protein turnover, chaperones (O). In Pfam analysis, most abundant domains identified were representing "Pkinase" followed by "Pkinase_Tyr" and "PPR_2". Comparative count of unigene annotatin using various databases are presented in Figure 2.

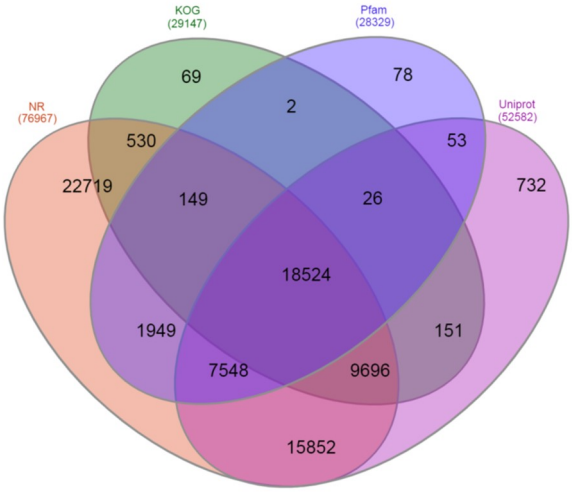
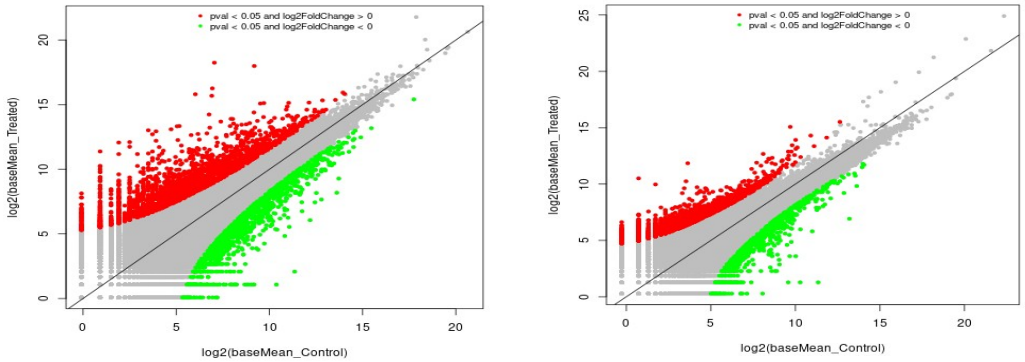


Figure 2. Venn diagram representing share of various databases used for annotation of Unigenes.

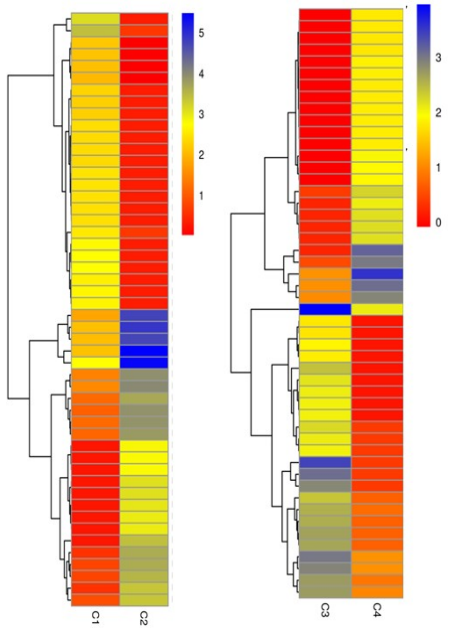
3.4. Differentially Expressed Transcripts

Analysis of transcripts derived from the leaves of stressed and control Kalpa Sree seedlings revealed that 2388 transcripts are significantly upregulated and 1278 are significantly downregulated whereas similar analysis of Kalpatharu transcripts showed significant upregulation and downregulation of 2868 and 778 transcripts, respectively (Figure 3a,b). Also, significantly expressed genes (i.e., highly up and downregulated genes) are represented in form of heatmap using pheatmap package of R, folowing hierarchical clustering approach (Figure 4a,b). Analysis of control and stressed Kalpa Sree leaf transcriptome showed significant upregulation of PHLOEM PROTEIN 2-LIKE A1-like (Log2 FC 11.19), WRKY transcription factor 40 isoform X1 (Log2 FC 9.59) and downregulation of glycerol-3-phosphate acyltransferase 3 transcripts. Significant upregulation of transcripts encoding polyamine oxidase (Log2 FC 9.78), arabinose 5-phosphate isomerase (Log2 FC 6.52), WRKY transcription factor 40 isoform X1 (Log2 FC 5.57) among others and downregulation of aquaporin PIP1-2 transcript (Log2 FC −1.8), ethylene responsive transcription factor ERF105 (Log2 FC −1.9) was documented in Kalpatharu leaves.



(**a**) (**b**)

Figure 3. Scatter plot representation of log2 (basemean-treated) values for transcripts expressed in control and treated leaf sampes of Kalpa Sree (**a**) and Kalpatharu (**b**).



(**a**) (**b**)

Figure 4. Unsupervised hierarchical clustering analysis of differentailly expressed transcripts. Heat Map showing clusters of differentially expressed genes in (**a**) Kalpa Sree (C1: control, C2: stressed) (**b**) Kalpatharu (KT) (C3: control, C4: stressed). The codes of colour bars are also presented.

Pathway analysis of unigenes divulged that in the primary metabolic pathways, carbohydrate and amino acids metabolism are enriched, and in the genetic information processing pathways, protein translation and degradation or turnover pathways are highly enriched than transcriptional changes, in environmental information processing signal transduction pathways are significantly enriched compared to membrane transport or signaling molecule interaction.

3.5. Genic Markers for Drought Tolerance

Genic simple sequence repeats (SSRs) or genic microsatellites or expressed sequence tag (EST)-SSRs are novel resources not only as a molecular markers but also to provides invaluable information regarding the adaptation of a species towards external stressors such as drougth or moisture stress. In this study, around 17,296 genic SSRs were identified from the transcriptome sequences of coconut with a potential for use in molecular breeding. The most common SSRs were di-nucleotide repeats (10,826) followed by tri-nucleotide repeats (5699); quad-, penta-, and hexa nucleotides are of 633, 89, and 49, respectively. Among the di-nucleotide repeats, GA/TC (1904) was most abundant, followed by CG/CG (1849), AG/CT (1803), TC/GA (1573) among others.

3.6. Differential Expression of Long Non-Coding RNAs

Analysis for expression of long non-coding RNAs (lncRNAs) in transcriptome data set revealed that a total of 63 Unigenes are potential lncRNAs. Differential expression analysis of those lncRNAs revealed that in Kalpa Sree 32 lncRNAs are differentially regulated whereas in Kalpatharu 59 lncRNAs are differentially regulated.

4. Discussion

Climate change is expected to increase mean annual temperatures, decrease the precipitation and sea water incursions in coastal and low lying areas. Plantation crops being perennials and having

relatively long gestation period to yield economic produce are under major threat to water-deficit stress due to decline in precipitation [9,10]. In this context, the traditional coconut growing regions are expected to see a shift in area coverage owing to reduced rainfall in future climate scenario [9]. Hence, it is pertinent to develop climate-smart coconut which can withstand water-deficit stress [11,12]. Unfortunately the genomic resources of coconut—an economically important plantation crop—is relatively poor. Accordingly this study was designed with an objective of identifying suitable genetic markers linked to water-deficit stress. We have chosen two coconut varieties Kalpa Sree (dwarf) and Kalpatharu (tall) of distinct morphoforms, having differential WUE and biochemical or physiological response in order to analyze their molecular response during water deficit stress utilizing RNA Seq approach. Our study has delineated that transcriptome response of both the varieties vary significantly as Kalpa Sree showed upregulation of genes involved in phloem protein like A1 and WRKY transcription factor 40 isoform X1 and downregulation of transcripts involved in metabolism. Kalpatharu on the other hand downregulated aquaporin PIP1-2 transcript and ethylene responsive transcription factor to tide over water deficit stress and to improve its WUE. The differential gene expression pattern is also corroborated by the differences in the enrichement of KEGG metabolic pathways and KOG analysis of the transcript data sets suggesting differential molecular and biochemical responses of the varieties.

5. Conclusions

Thus this investigation elucidates the genotype-specific differential molecular responses of an economically important plantation crop during water deficit stress for which no prior genome-level response for water-deficit stress was available. Further the EST-derived SSRs uncovered in this study would add to the much needed molecular marker data set for developing climate-smart coconut. It also underscores the importance of lncRNAs repertoire in shapping up the adaptive response of coconut to water stress. To the best of our knowledge this is the first report of whole transcriptome analysis of coconut subjected to water deficit stress.

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Abbreviations

ASM	Available soil moisture
BLAST	Basic local alignment search tool
ECW	Epicuticular wax
KAAS	KEGG automatic annotation server
Nr	non-redundant
SSR	Simple sequence repeats
WTS	Whole transcriptome sequencing
WUE	Water use efficiency

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