Eur J Plant Pathol (2018) 151:173–193
https://doi.org/10.1007/s10658-017-1365-8

Comparative transcriptome profiling of healthy and diseased Chowghat Green Dwarf coconut palms from root (wilt) disease hot spots

M. K. Rajesh · K. E. Rachana · K. Kulkarni · B. B. Sahu · R. J. Thomas · A. Karun

Accepted: 11 October 2017 / Published online: 16 October 2017
© Koninklijke Nederlandse Planteziektenkundige Vereniging 2017

Abstract Understanding the molecular basis of host-pathogen interactions is imperative for devising disease management strategies. The root (wilt) disease is the foremost debilitating disease threatening coconut production in India. To explore the molecular mechanisms involved in compatible and incompatible interactions, global transcriptome profiling of leaves of healthy and diseased Chowghat Green Dwarf (CGD) palms was conducted. RNA-Seq analysis generated more than 190 million 100 bp reads from both healthy and diseased samples. Assembled transcriptome yielded 59,282 transcripts with a median length of 987 bp. BLASTX annotation of transcriptome resulted in 39,665 transcripts getting annotated from Uniprot and date palm proteome database. Differential gene expression profiling analysis revealed 2718 transcripts to be up- or down- regulated in the diseased palms in comparison to healthy control at a fold change of 2 and above with a *p* value <=0.05. The differentially expressed transcripts could be categorized into pathways which included cell wall biogenesis, primary and secondary metabolism, plant-pathogen interaction, cellular transport, hormone biosynthesis and signaling. Validation by quantitative real time PCR (RT-qPCR) of a set of arbitrarily selected genes, both up-regulated and down-regulated, established a comparable pattern as observed by RNA-Seq analysis. Overall, the resources generated in this study provide an in-depth analysis and new insights into the interaction of coconut palms with the root (wilt) disease pathogen.

Keywords *Cocos nucifera* · Root (wilt) disease · Gene expression · RNA-Seq · Quantitative PCR

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10658-017-1365-8) contains supplementary material, which is available to authorized users.

M. K. Rajesh (✉) · K. E. Rachana · A. Karun
ICAR-Central Plantation Crops Research Institute, Kasaragod, Kerala 671124, India
e-mail: rajesh.mk@icar.gov.in

K. Kulkarni
Bionivid Technology Private Limited, Bengaluru, Karnataka 560043, India

B. B. Sahu
National Institute of Technology, Rourkela, Odisha 769008, India

R. J. Thomas
ICAR-CPCRI (RS), Kayamkulam, Kerala 690533, India

Introduction

The coconut palm, *Cocos nucifera* L. (Arecaceae; 2n = 32) is distributed throughout the tropical and sub-tropical zones of the world. A versatile palm known as the 'tree of life', almost all of its parts find use in domestic, commercial or industrial sectors. The tender coconut water, which is the liquid endosperm, is one of the most nourishing and wholesome natural drinks available. Coconut palms are susceptible to numerous diseases- some of these diseases progressively reduce the vigour of the palm, resulting in severe yield losses; others are lethal. The root (wilt) disease is the main disease threatening coconut production and productivity

in India. This debilitating disease first made its appearance in Southern Kerala State of India sometime between 1868 and 1878 and has spread from the epicenter of infection to other areas since then (Koshy 1999). The major distinguishing symptoms of the disease include flaccidity of the leaflets, followed by yellowing and necrosis. Etiological studies have suggested the association of phytoplasma (*Candidatus* phytoplasma oryzae, 16Sr XI sub group) with the root (wilt) disease (Solomon et al. 1983; Manimekalai et al. 2010). Since phytoplasmal diseases cannot be controlled by conventional plant protection measures, development of disease resistant/tolerant cultivars offers the only a sustainable disease management option, which can be effective in the long run given the perennial nature of coconut. The identification of root (wilt) disease-resistant genotypes in a population would significantly contribute towards developing root (wilt) disease-resistant cultivars possessing various sources of resistant genes.

The biology of coconut palm and the association of phytoplasma with the disease cause hindrance in screening of coconut cultivars for resistance to root (wilt) disease under controlled conditions. Field level evaluation of germplasm for identifying genotypes resistant/tolerant to phytoplasma has been reported earlier in sesame (Singh et al. 2007). Evaluation of the field grown palms for disease symptoms has been utilized as an alternate method of locating resistance/tolerance trait in coconut to phytoplasmal diseases like the lethal yellowing disease of Caribbean and African countries (Gurr et al. 2016), Cape Saint Paul Wilt Disease in Ghana (Quaicoe et al. 2009), yellow decline in Malaysia (Nejat et al. 2009) and Weligama coconut leaf wilt disease in Sri Lanka (Perera et al. 2015). Results of a screening trial undertaken by Jacob et al. (1998) revealed that none of the coconut cultivars/hybrids evaluated possessed the desired level of resistance to root (wilt) disease. As an extension of this study, a comprehensive breeding programme was undertaken for identification of field level resistance to root (wilt) disease in coconut cultivars and involved survey and selection of mother palms in farmers' fields in four disease endemic districts of Kerala state, India (Nair et al. 1996). These surveys revealed that 75% of Chowghat Green Dwarf (CGD) palms in farmers' fields in the disease hot spots were free from root (wilt) disease. Thus, there was an indication of the existence of enhanced level of resistance in CGD palms to root (wilt) disease (Nair et al. 2004) and the potential of host plant resistance to contribute to management of root (wilt) disease through development of disease resistance varieties through selection and hybridization.

Limited genomic resources are available for coconut, which restricts crop improvement programmes. Being a genome independent technique, RNA-Seq is highly useful for analyzing the transcript of non-model organisms (Wang et al. 2009; Nagalakshmi et al. 2010) like coconut. With focus only on the transcribed portion of the genome, RNA-Seq circumvents repetitive and non-coding sequences, which constitute the bulk of eukaryotic genomes (Ozsolak and Milos 2011). Results from RNA-Seq experiments have furthered understanding of complexities of gene transcription patterns, gene structural variants and gene regulatory networks (Wang et al. 2009). RNA-seq profiling has been utilized to provide comprehensive transcriptomics landscapes and insights into the molecular pathways during the host-phytoplasma interactions during witches' broom disease of Mexican lime trees (Mardi et al. 2015), flavescence dorée phytoplasma in grapevine (Abbà et al. 2014), witches' broom disease of Paulownia (Liu et al. 2013; Fan et al. 2014, 2015) and apple (Siewert et al. 2014), peanut witches'-broom phytoplasma in *Catharanthus roseus* (Liu et al. 2014) and Huanglongbing disease of citrus (Wang et al. 2016). RNA-Seq studies have been undertaken previously in coconut to identify factors involved in RNA-directed DNA methylation (Huang et al. 2014), response of coconut leaves to yellow leaf decline infection (Nejat et al. 2015), to identify transcripts expressed in embryogenic calli (Rajesh et al. 2016) and gene conferring 'pandan-like' aroma (Saensuk et al. 2016).

The knowledge available on the molecular events invovled in the establishment of resistance or susceptibility of coconut palms to root (wilt) disease is quite limited. A deep understanding of response of coconut palms to root (wilt) disease will definitely provide a fillip to elucidating mechanisms of palm-pathogen interaction and possibly develop newer strategies for pathogen control and development of disease resistant varieties. As a first step, in this study, we have employed Illumina paired-end sequencing technology to perform RNA-Seq of leaves of healthy and root (wilt) diseased CGD coconut palms. Studies on the breeding behaviour of CGD palms have indicated that there is total overlapping of the male and female phases in CGD, because of which these palms are completely self-pollinated; also, microsatellite genotyping have revealed the genetic

uniformity of these palms (Thomas et al. 2015). To test the hypothesis that gene expression was differentially regulated in healthy and root (wilt) diseased palms, *de novo* assembly, gene annotation, pathway assignment and differential gene expression analysis was undertaken in this study by comparing the transcripts of healthy and root (wilt) diseased CGD palms. The results obtained in this study uncovered, for the first time, a number of differentially expressed transcripts in coconut, responsive to root (wilt) disease.

Materials and methods

Plant materials

Chowghat Green Dwarf (CGD) palms, maintained in the farm at ICAR-CPCRI (RS), Kayamkulam, Kerala State, India, which is a disease hot spot area, were taken for the study. These palms, planted during June 1998, have come to flowering after three years (during September–December 2001). The criteria given by Nair et al. (1996) were followed for the selection of healthy CGD palms. Samples were collected from both healthy and diseased palms from the same plot (Fig. 1 a, b). The disease severity was assessed using the indexing method developed by Nambiar and Pillai (1985). The root (wilt) disease affected palms in the disease advanced category, with disease index of 59.5, 66.25 and 62.5, were used in the analysis. The healthy and diseased palms were tested serologically (Sasikala et al. 2010) to ascertain that they were free of or were infected with root (wilt) disease respectively.

RNA extraction

Spindle leaf tissues were sampled from three each of healthy and diseased palms. The samples were snap-frozen in liquid nitrogen immediately and extraction of total RNA was carried out using the Trizol reagent (Invitrogen). Treatment with DNase I (Fermentas) was performed according to the manufacturer's instructions. Assessment of the quality and purity of the extracted RNA was undertaken based on ratio of absorbance at 260 and 280 nm (A260/280) and RNA integrity number (RIN) was determined by using a Bioanalyzer (Agilent Technologies).

Transcriptome sequencing

Equal amounts (10 μg) of total RNA, independently isolated from three each of healthy and diseased CGD samples, were pooled together to form the healthy and diseased bulk respectively. About 10 μg of total RNA from both the bulks were then utilized in the preparation of the RNA-seq library by means of TruSeq RNA Sample Prep Kits (Illumina) according to the manufacturer's instructions. Briefly, poly $(A)^+$ mRNA molecules were enriched from 10 μg of total RNA using poly-T oligo-attached magnetic beads, following which the mRNA was fragmented into 200–700 nt fragments using divalent cations at 94 °C for 5 min. These fragments were then utilized for the synthesis of first strand cDNA using reverse transcriptase and random hexamer primers. This was followed by second strand cDNA synthesis utilizing DNA polymerase I and RNase H. End repair and phosphorylation of the synthesized cDNA fragments were then carried out. Later, 3′-adenylated of the repaired cDNA fragments were performed and adapters were ligated to the ends of the 3′-adenylated cDNA fragments. The requisite fragments were purified by agarose gel electrophoresis and PCR-enriched to obtain the final cDNA library. At every step, bioanalyzer plots were utilized to assess the quality of mRNA, success of enrichment and also the sizes of fragments and the final library. Additionally, gel electrophoresis was employed to establish the size distribution of the sequencing library. Before sequencing, the quantity of the library was assessed by RT-qPCR. After the libraries were constructed, paired end run was carried out on an Illumina HiSeq 2000 platform at Scigenom, India.

De novo transcriptome assembly and evaluation

Comprehensive transcriptome assembly was as carried out as per the protocol of Bankar et al. (2015). In brief, prior to performing assembly, the fastq files were trimmed, by removing the first 15 bases using a Perl script program, to circumvent specific sequence bias. Additionally, reads possessing average base quality score < Q20 Phred score were excluded from the analysis. The reads from healthy and diseased samples, after filtering for low quality reads, were first combined to generate the coconut reference transcriptome using the *De Bruijn* graph based Trinity Assembler (Henschel et al. 2012). The trimmed reads of healthy and diseased samples were then individually

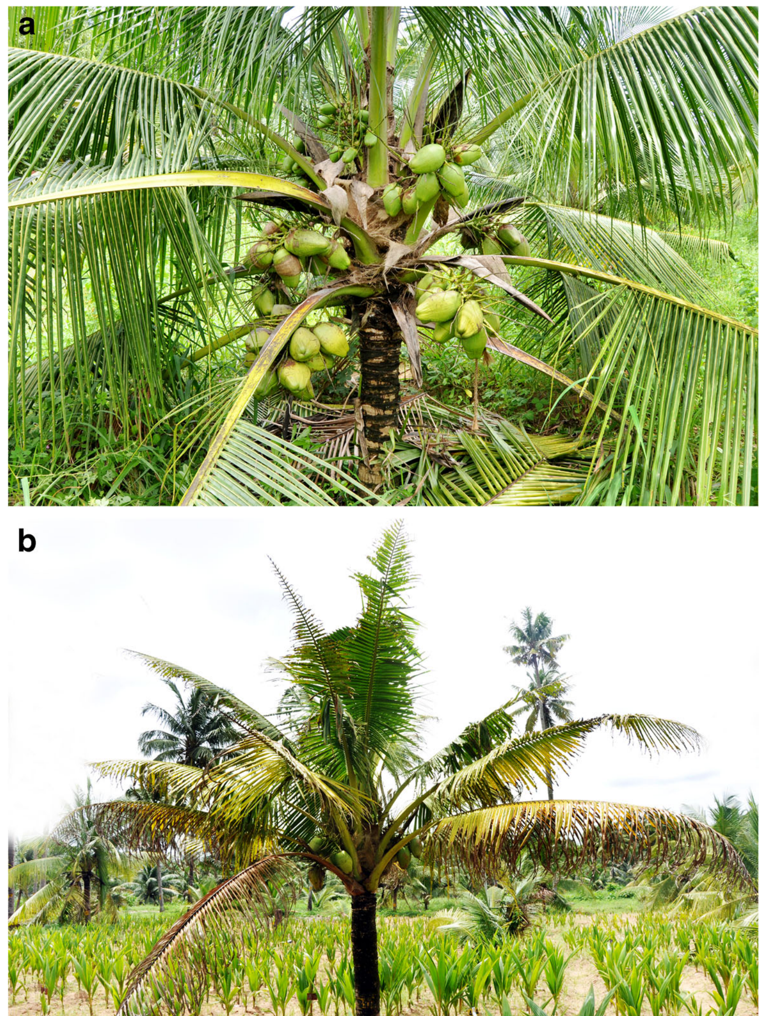

Fig. 1 Healthy (**a**) and root (wilt) diseased (**b**) CGD palms

aligned to the assembled transcriptome (length > = 150 bp) using BOWTIE program. Upto 3-mismatches in the seed region (length = 50 bp) were allowed and all multiple mapped position were reported. As an extension to the protocol recommended, clustering of the resultant transcripts was also done to arrive at the finer non-redundant transcriptome.

Gene expression estimation and differential gene expression

DESeq software (Anders and Huber 2010) was employed for normalization and expression profiling. Transcripts, with base mean value and read count ≥10, were considered as 'expressed'. Sample-specific expression of transcripts was identified using the above criteria. Transcript expression and normalization was evaluated by creating scatter plot between healthy and diseased reads. Differential expression of transcripts was determined by applying a stringent cut off of 2-fold and above as upregulated (FC + 2.0 and above) and −2-fold and below as downregulated (FC - 2.0 and below) with a $p \leq 0.05$. Calculation of fold change in expression was based on number of reads mapped and RPKM (reads per kilobase of transcript per million mapped reads). Unsupervised hierarchical clustering of differentially expressed genes (DEGs) was done using Pearson Uncentered Algorithm with average linkage rule using Cluster 3.0 (De Hoon et al. 2004) and visualized using Java Tree View (Saldanha 2004).

Functional annotation and classification

The assembled and expressed transcripts were annotated by performing the following steps: (i) comparison with Uniprot plant proteins database using BLASTx program (retaining only those matches with e-value ≤1e-5 and similarity score ≥ 40% for further annotation), and (ii) predicted proteome from date palm genome database (http://qatar-weill.cornell.edu/research/datepalmGenome/). Gene ontology and pathway annotation was obtained using Blast2GO tool and also from date palm genome database.

BAN (Biological Analysis Network) modeling of disease phenotype

Statistically significant and enriched gene ontologies (GO) and pathways, along with the differentially expressed genes and genes which were uniquely expressed, was used as input for 'Bridge Island' Software (Bionivid Technology Pvt. Ltd., India) for the identification of key edges that connect GO/Pathways. The statistical scores, derived from differential expression and biological analyses, were employed as attributes for visualizing networks. The output obtained from Bridge Island Software was utilized as input in CytoScape V 2.8 (http://www.cytoscape.org/). Force Directed Spring Embedded Layout algorithm was used for visualizing the networks which encompassed biological categories and differentially expressed genes, which were significantly enriched. Additionally, to this core regulatory network, all the genes were colored on the basis of their fold change in diseased in comparison to healthy sample.

Gene validation and expression analysis by quantitative real-time PCR (RT-qPCR)

Selected DEGs were validated using RT-qPCR. Primers were designed using Primer 3.0 software (http://primer3.ut.ee/) for 29 differentially expressed genes which were randomly selected *i.e.* 15 of upregulated and 14 downregulated in diseased CGD palms compared to healthy palms. The sequence of the primers, the length of the amplicon and annealing temperature for each primer pairs are presented in Supplementary Tables 1 and 2. Total RNA was isolated from five each of healthy and diseased CGD palms from the same plot at the ICAR-CPCRI (RS), Kayamkulam, Kerala State, India, as described earlier. The integrity, concentration and quality check of the extracted RNA were carried out. Synthesis of cDNA was performed using High Capacity RNA-to-cDNA kit (Applied Biosystems).

To verify the specificity of the primer sets, PCR was carried out using pooled cDNA as template. The amplicons were separated by 2% (*w*/*v*) agarose gel electrophoresis and bands were excised from the gel, purified using QIAquick Gel extraction kit (Qiagen) and sequenced.

Aliquots for quantitative RT-PCR (RT-qPCR) reactions included 12.5 μL of 2X SYBR Green PCR Master Mix (Applied Biosystems), 1 μL each of forward and reverse primers (Sigma), 1 μL of template (10× diluted cDNA from samples) and 10.5 μL of sterile distilled water made upto a final volume of 25 μL. PCR was performed as follows: initial holding stage 52 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and a final step at 60 °C for 1 min. All reactions were performed in triplicate in 48-well reaction plates using Step One Real Time PCR machine (Applied Biosystems). The housekeeping gene α-tubulin was used as endogenous control (forward primer: 5′-CTGG TGTCCTACTGGCTTC-3′; reverse primer: 5′-GACCATGATTACGCCAAG -3′) (Rajesh et al. 2014). Three technical replicates were included for each biological replicate. No template controls were also included for each gene to detect any spurious signals arising from amplification of any DNA contamination or primer dimer formed during the reaction.

A relative quantification method was used to determine the gene expression levels. The expression of genes of interest was normalized to that of α-tubulin. The statistical significance of the differences ($p < 0.05$) in target mRNA expression level were analyzed with the Relative Expression Software Tool (REST®, Qiagen Inc., USA). The group-wise comparisons and the statistical analyses of relative expression results were calculated (Pfaffl et al. 2002) using Pair Wise Fixed Reallocation Randomisation Test©.

Results

Transcriptome sequencing and *de novo* assembly

Approximately 111 million and 119 million 101-bp clean paired end reads were obtained for the healthy

[NCBI Short Read Archive (SRA) accession number SRX436961] and diseased samples (SRA accession number SRX437650) respectively. More than 80% of reads had Q30 or more Phred score (error probability ≥0.01). The average GC content was 47.02%. After trimming and filtering for low-quality reads, a total of 102,579,213 (~17.64 Gb) and 110,905,757 (~19.07 Gb) reads were obtained from the healthy and diseased libraries respectively with an average GC content of 46%. The trimmed reads then were subjected to pooled *de novo* transcriptome assembly. Primary transcriptome assembly was validated and refined before subjecting to annotation. A total of 186,637 transcripts were obtained in primary assembly and after further amelioration and clustering, it resulted in 59,282 non-redundant transcripts (unigenes) (Suppl. Table 3). The maximum sequence length was 19,292 bp, the average sequence length, 1386 bp, and N50 was 2180 bp. Assembly statistics of coconut leaf transcriptome is given Supp. Table 4. Length distribution analysis showed that more than 50% of the transcripts were between 200 and 1000 nt long (Suppl. Fig. 1).

Functional annotation using homology searches

Protein sequences pertaining to plant kingdom was obtained from Uniprot database for transcriptome annotation. BLASTX was performed against the protein database and one best hit was selected based on E-value (<=0.001), percentage identity (> = 30), query coverage (> = 50) and bit score (> = 35). While 37,748 (63.6%) transcripts showed significant matches to known proteins in Uniprot, 21,534 (36.2%) transcripts did not show any significant matches to known proteins. Additionally, the 59,282 transcripts detected were subjected to BLASTX with date palm proteome comprising of 19,414 proteins. A total of 33,757 (56.9%) could be annotated with 12,184 date palm proteins, covering 62% of the date palm proteome (Suppl. Table 5).

Expression profiling of assembled transcriptome

Transcript ID along with read count was used as an input for normalization, quantitation and differential expression analysis using DESeq. Normalized transcriptome data showed a high degree of correlation (0.89) in the expression levels of transcripts from both healthy and diseased samples (Fig. 2). Unsupervised hierarchical clustering of differentially expressed transcripts showed distinct pattern of expression signature between healthy and diseased palms (Fig. 3).

A total of 136 transcripts were expressed specific to diseased sample (Suppl. Table 6) and 454 transcripts were specific to healthy sample (Suppl. Table 7). A total of 2718 transcripts were differentially expressed (up or down regulated) in the healthy and diseased samples (Fig. 4; (Suppl. Tables 8 and 9), while 55,974 transcripts were commonly expressed. A list of representative transcripts that were differentially expressed among the healthy and diseased genotypes with respect to metabolic pathways, cell-wall fortification, transcription factors, signaling and transporter families is provided in Table 1.

Gene ontology, enrichment and pathway analysis

Annotated transcripts, that were specific to healthy and diseased leaf samples along with those that were differentially regulated, were enriched with Gene Ontology (GO) and pathway information from Uniprot and KEGG pathway databases respectively. Further, they were subjected to statistical enrichment analysis to calculate the Z score for each in the GO and Pathways. Top 20 GO and pathways based on harboring most of the genes are summarized in Figs. 5 and 6. Those GO and pathways, with a Z score of >2, were considered as significantly deregulated and subjected to downstream regulatory network analysis. A total of 54 GO categories and 12 pathways were enriched with significant score. Gene ontology analysis of differentially expressed transcripts was performed again with GO annotations obtained from date palm genome database (Suppl. Fig. 2 a, b, c). It was seen that key enriched GO terms obtained from date palm database matched with homology-based GO annotation.

Gene regulatory network underlying healthy and diseased phenotypes

Statistically significant enriched GO and pathways along with specifically expressed and differentially expressed genes were provided as input to Bridge Island software, which identifies and enriches the key connections and calculates enrichment score for nodes (Genes / GO / Pathways) and provides the resultant file. This file was used as an input to Cytoscape V 2.8.3 to visualize the regulatory network and connections. Circular layout algorithm was applied on to the nodes and edges. Nodes were sized based on their connectivity score and colored

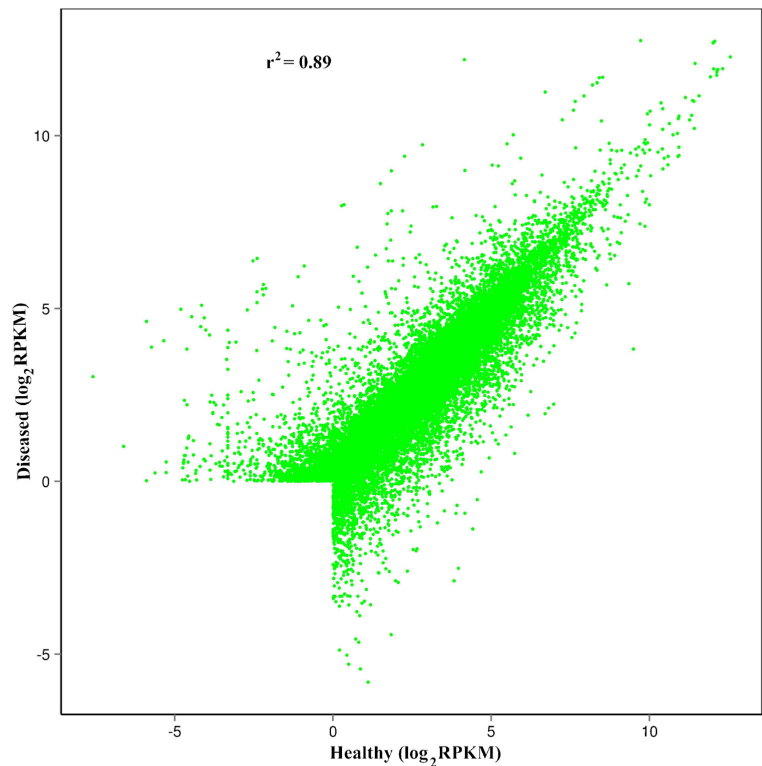

Fig. 2 Scatter plot representation of $\log_2$ (RPKM) values for transcripts expressed in healthy and diseased samples. Pearson's correlation coefficients (r^2) between the two transcriptomes are presented

based on their fold change/sample specific expression status (Fig. 7). Thus modeled gene regulatory network underlying healthy and disease phenotypes showed significant enrichment of key GO and Pathways including metabolic pathways, metal ion binding [GO:0046872], biosynthesis of secondary metabolites, integral component of membrane [GO:0016021], chlorophyll binding [GO:0016168], protein-chromophore linkage [GO:0018298], carbon metabolism, photosynthesis, light harvesting [GO:0009765], chloroplast [GO:0009507], photosystem I [GO:0009522], photosystem II [GO:0009523], carbon fixation in photosynthetic organisms, biosynthesis of antibiotics and photosynthesis - antenna proteins to be critically deregulated in healthy and disease phenotypes. Most of the significantly enriched genes were annotated as uncharacterized protein and some of the characterized proteins, which were enriched, included chlorophyll a-b binding 4, chloroplastic-like protein, fructose-bisphosphate aldolase [EC 4.1.2.13], peroxidase [EC 1.11.1.7], pectinesterase [EC 3.1.1.11], polygalacturonase, putative [EC 3.2.1.67] and metabolism related enzymes (Table 2). Calmodulin-like 41, putative and WRKY DNA-binding protein 33 isoform 1, which are characteristic of plant-pathogen interaction pathways, was notably up regulated, though the pathway was not statistically enriched.

Validation by RT-qPCR

In order to validate the RNA-Seq results, we confirmed the differential expression of 29 selected genes, representing different categories, by real- time PCR analysis. All DEGs tested produced unique PCR amplicons confirming the assembly. The RT-qPCR results showed that the expression trends of all these genes were consistent with RNA-Seq data (Suppl. Table 1 and 2), confirming the reliability of the transcriptome analysis.

Discussion

In order to obtain a comprehensive understanding of host-pathogen interactions, it is imperative to gain a thorough knowledge about the coordinated expression of different defense gene networks that govern a particular interaction. In this study, the plant defense response

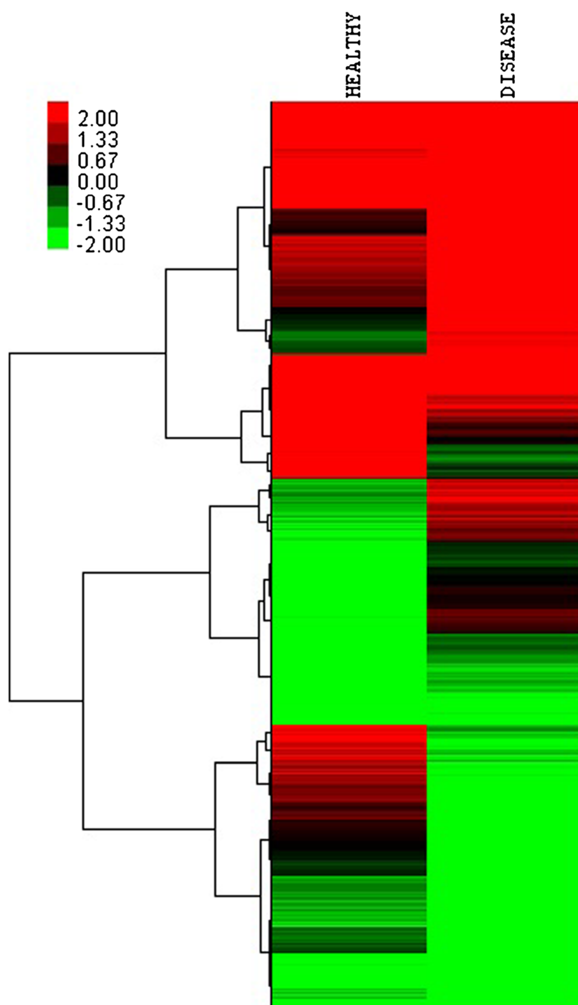

Fig. 3 Unsupervised hierarchical clustering analysis of differentially expressed transcripts. Heat map showing gene expression clusters. Each line refers to data of one gene. The colour bar represents log2 of fold change values, ranging from green (−2) to red (2)

in coconut palms, following infection by root (wilt) disease, was investigated by a comparison of expression patterns of various genes between healthy and diseased palms using next generation RNA-Seq technology. Most of the upregulated transcripts identified were found to be among reported genes in other crops that are involved in defense-related functions and metabolic pathways.

DEGs related to metabolic pathways

We observed that the transcripts involved in carbohydrate, amino acid, lipid and phenylpropanoid metabolism were differentially expressed in diseased CGD palms as compared to healthy palms. Plant metabolic pathways are generally perturbed during infection of phytopathogens. It is a known fact that plant's adaptive defence mechanism against pathogen attack is an energy intensive process. Defense responses are multipronged and entail diversion of energy and primary metabolites towards biochemical pathways that counteract pathogen invasion (Berger et al. 2007; Kangasjärvi et al. 2012; Rojas et al. 2014; Scheideler et al. 2002). The general agreement is that the creation of a conducive energy balance for defense occurs by the compensation of upregulation of plant defense pathways by downregulation of plant metabolic pathways (Rojas et al. 2014).

Rojas et al. (2014) have suggested that the regulation of genes involved in the primary metabolism appears to be rather complicated- they could be up- or down-regulated, even though they were invovled in the same pathways. The high expression of the genes involved in carbohydrate metabolism might be because of the transcriptional regulation controlled by the pathogen effectors; data generated from previous transcriptomics studies have suggested that the expression of genes involved in carbohydrate metabolism is stimulated by avirulent pathogens or pathogen-derived elicitors, which in turn mediates downstream defense responses, with the regulation being operated at multiple levels (Rojas et al. 2014). The results obtained in this study corroborate earlier studies reporting differential expression of genes involved in carbohydrate metabolic process due to attack by phytopathogens (Rojas et al. 2014). Endo-1, 4-β-mannosidase, an enzyme involved in the degradation of hemicelluloses, was specifically expressed in diseased CGD palms (c96167_g1_i1). This enzyme catalyses the breakdown of mannan, a component of hemicelluloses present in plant cell wall, and has been implicated as playing a key role in the virulence of phytopathogens by facilitating degradation of host cell walls (Beliën et al. 2006; Mardi et al. 2015).

In our study, 3-ketoacyl-CoA synthase, a component of a fatty acid elongase complex involved in fatty acid biosynthesis, was found to be up-regulated in diseased CGD palms. Very long chain fatty acids (VLCFA) are obtained by the repetitive addition of C2 units, provided by malonyl-CoA, to an acyl-CoA by the 3-ketoacyl-CoA synthase (Blacklock and Jaworski 2006). Lipid signaling is a vital component of plant resistance mechanisms against pathogens, with probable mechanistic

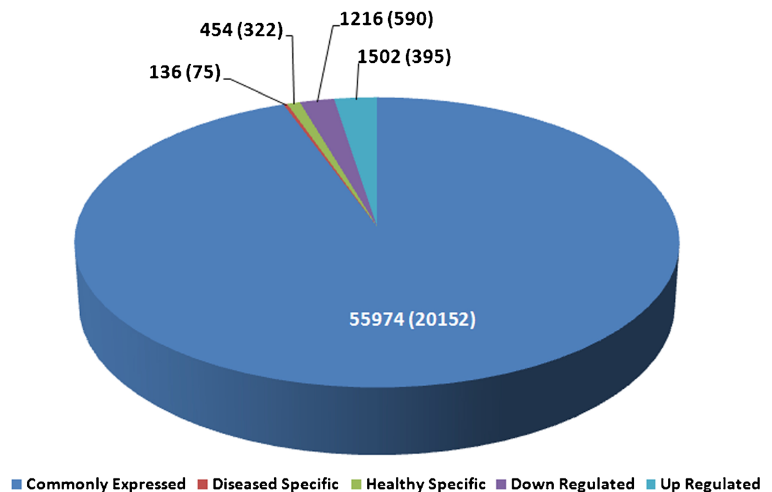

Fig. 4 Distribution of transcripts based on their expression levels in healthy and diseased palms. Numbers in parenthesis indicate unannotated transcripts

interaction with SA-dependent immunity (Zhang and Xiao 2015). VLCFA pathway has been associated to biotic stress responses as these fatty acids are involved in the biosynthesis of the plant cuticle (Raffaele et al. 2009) and the generation of sphingolipids, which have been associated with regulation of SA accumulation in plant cells (Sánchez-Rangel et al. 2014).

With respect to biosynthesis of amino acids, transcripts encoding glutamine synthase and asparagine synthetase were down regulated in the leaves of CGD palms compared to healthy palms. Earlier studies using transcriptomic and metabolomic approaches have revealed the regulation of numerous genes implicated in amino acid biosynthesis in response to attack by phytopathogens (Scheideler et al. 2002; Ward et al. 2010), pointing towards a role for metabolism of amino acids in defense responses (Zeier 2013). Build-up of amino acids or their metabolic byproducts could trigger resistance responses against phtyopathogens, which could even act independent of SA- and ROS-mediated defense pathways (Rojas et al. 2014). Seifi et al. (2014) had revealed the key role played by asparagine synthetase in the immune response of tomato against *Botrytis cinerea*. A dual role was speculated for the enhanced induction of asparagine synthetase in susceptible tomato plants– firstly, in its involvement in the pathogenicity of *B. cinerea* by both assisting host senescence initiated by the phytopathogen and secondly, making available an abundant reservoir of nitrogen to facilitate and support growth of the pathogen *in planta* (Seifi et al. 2014). The role of glutamine metabolism in plant defense against phytopathogens has also been reported (Seifi et al. 2013). Alterations in the amino acid metabolism, occurring in the plant host as a response against pathogens, can also modulate crosstalk of SA- and JA-regulated defence pathways (Zeier 2013).

Our analysis revealed that key enzymes involved in phenylpropanoid biosynthesis pathway were differentially expressed between healthy and diseased CGD palms. Various biotic stimuli are known to trigger the biosynthesis of phenylpropanoids such as flavonoids, tannins, lignins, lignans, stilbenes, hydroxycinnamic acids, phenols, catecholamines, phenolic and phenylpropanoic acids (Ferrer et al. 2008; Vogt 2010). Many of these phenylpropanoids have antioxidant activities and have crucial role in maintaining redox homeostasis in the host during pathogen invasion. Activation of genes of phenylpropanoid pathway has been reported as an early response to *Erwinia carotovora* subsp. *carotovora* infection in Chinese cabbage (Zhang et al. 2007), *Fusarium* attack in flax plants (Kostyn et al. 2012) and in *Camelina sativa* in response to the *Sclerotinia sclerotiorum*, which is a necrotrophic fungus (Eynck et al. 2012). Chalcone synthase (CHS), belonging to the plant polyketide synthase superfamily, provides the starting material for a variety of flavonoids, which possess important, but diverse roles (Sanchez 2008). Transcripts encoding CHS were either specifically expressed in the leaves of the healthy palms or unregulated in them. Flavonoids are known to play an important role in plant defense, and CHS, which catalyses the first committed step of the branch of the phenylpropanoid pathway leading to flavonoid biosynthesis, assays a key role in the regulation of this pathway (Dao et al. 2011). Similar to the results obtained in this study, genes invovled in flavonol biosynthesis were

Table 1 Representative transcripts that were differentially expressed among the healthy and diseased genotypes

Sl. No.	Transcript ID	Regulation	Log2 fold change	*P*-value	Orthologous ID	Gene Name	GO		
							Biological function	Molecular function	Cellular
A. DEGs related to metabolic pathways									
1.	c96167_g1_i1	diseased specific	–	0.24	W9QQT1_9ROSA	mannan endo-1,4-β-mannosidase 2	carbohydrate metabolic process [GO:0005975]	hydrolase activity, hydrolyzing O-glycosyl compounds [GO:0004553]	N/A
2.	c33066_g1_i1	healthy specific	–	0.00	A7LP06_ELAOL	naringenin-chalcone synthase	biosynthetic process [GO:0009058]	transferase activity, transferring acyl groups other than amino-acyl groups [GO:0016747]	N/A
3.	c79328_g1_i1	down regulated in diseased palms	−4.81	2.05E-06	B3TLZ2_ELAGV	asparagine synthetase	asparagine biosynthetic process [GO:0006529]	asparagine synthase (glutamine--hydrolyzing) activity [GO:0004066]	N/A
4.	c114629_g1_i1	down regulated in diseased palms	−2.08	9.1E-03	A0A0B4-V112_BOENI	glutamine synthetase	glutamine biosynthetic process [GO:0006542]	ATP binding [GO:0005524]; glutamate-ammonia ligase activity [GO:0004356]	N/A
5.	c79970_g1_i1	upregulated in diseased palms	2.40	0.00	A0A0A0L-X26_CUCSA	3-ketoacyl-CoA synthase	fatty acid biosynthetic process [GO:0006633]	transferase activity, transferring acyl groups other than amino-acyl groups [GO:0016747]	membrane [GO:0016020]
B. DEGs related to cell wall fortification									
6.	c39833_g2_i1	up-regulated in diseased palms	2.87	0.00	A0A061DR-W7_THECC	pectinesterase (EC 3.1.1.11)	cell wall modification [GO:0042545]	aspartyl esterase activity [GO:0045330]; enzyme inhibitor activity [GO:0004857]; pectinesterase activity [GO:0030599]	cell wall [GO:0005618]
7.	c119056_g1_i1	diseased specific	–	0.12	M0RTY6_MU-SAM	pectinesterase (EC 3.1.1.11)	cell wall modification [GO:0042545]	aspartyl esterase activity [GO:0045330]; enzyme inhibitor activity [GO:0004857]; pectinesterase activity [GO:0030599]	cell wall [GO:0005618]

Table 1 (continued)

Sl. No.	Transcript ID	Regulation	Log2 fold change	*P*-value	Orthologous ID	Gene Name	GO		
							Biological function	Molecular function	Cellular
8.	c2332_g1_i1	healthy specific	–	0.16	A0FHB9_LYCAU	pectinesterase (EC 3.1.1.11)	cell wall modification [GO:0042545]	aspartyl esterase activity [GO:0045330]; enzyme inhibitor activity [GO:0004857]; pectinesterase activity [GO:0030599]	cell wall [GO:0005618]
9.	c4735_g1_i1	healthy specific	–	0.00	B9SP63_RICCO	pectinesterase (EC 3.1.1.11)	cell wall modification [GO:0042545]; pectin catabolic process [GO:0045490]	aspartyl esterase activity [GO:0045330]; enzyme inhibitor activity [GO:0004857]; pectinesterase activity [GO:0030599]	cell wall [GO:0005618]
C. DEGs related to transcription factors									
10.	c29333_g1_i1	diseased specific	–	0.08	M7ZLA8_TRIUA	transcription factor RF2a	N/A	sequence-specific DNA binding [GO:0043565]; sequence-specific DNA binding transcription factor activity	N/A
11.	c45098_g2_i1	healthy specific	–	0.00	B3TLL1_ELAGV	MYB transcription factor MYB92	N/A	chromatin binding [GO:0003682]; DNA binding [GO:0003677]	N/A
12.	c22426_g1_i2	down regulated in diseased palms	−2.401	0.00	A0A061EHU2_THECC	NAC domain protein, IPR003441	flower development [GO:0009908]; innate immune response [GO:0045087]; leaf senescence [GO:0010150]; multidimensional cell growth [GO:0009825]; response to bacterium [GO:0009617];	DNA binding [GO:0003677]; sequence-specific DNA binding transcription factor activity [GO:0003700]	nucleus [GO:0005634]
13.	c38031_g1_i1	down regulated in diseased palms	−5.10	2.63E-05	A0A061EM50_THECC	WRKY DNA-binding protein 72	defense response [GO:0006952]	sequence-specific DNA binding [GO:0043565]; sequence-specific DNA binding	N/A

Table 1 (continued)

Sl. No.	Transcript ID	Regulation	Log2 fold change	*P*-value	Orthologous ID	Gene Name	GO		
							Biological function	Molecular function	Cellular
15.	c44929_g1_i1	down regulated in diseased palms	−3.762	1.31E-06	A0A061FWI7_ THECC	NAC domain protein, IPR003441	regulation of transcription, DNA-templated [GO:0006355]; transcription, DNA-templated [GO:0006351]	transcription factor activity [GO:0003700] DNA binding [GO:0003677]	nucleus [GO:0005634]
D. DEGs related to signaling									
16.	c23220_g1_i2	diseased specific	–	0.04	M8AQ39_AEGTA	LRR receptor-like serine/threonine--protein kinase	N/A	kinase activity [GO:0016301]	N/A
17.	c138193_g1_i1	diseased specific	–	0.24	G7LHV1_ MEDTR	histidine kinase	auxin polar transport [GO:0009926]; cell fate specification [GO:0001708]; embryo development [GO:0009790]; leaf vascular tissue pattern formation [GO:0010305]; root development [GO:0048364]; stem cell maintenance [GO:0019827]	kinase activity [GO:0016301]	nucleus [GO:0005634]
18.	c75260_g1_i1	healthy specific	–	0.00	D0EUY7_ MEDSA	Ca^{2+} and calmodulin--dependent protein kinase	N/A	ATP binding [GO:0005524]; calcium ion binding [GO:0005509]; protein kinase activity [GO:0004672]	N/A
E. DEGs related to transporter families									
19.	c61339_g1_i1	diseased specific	–	0.12	A0A0B0PBG6_ GOSAR	phosphate transporter PHO1-like	N/A	N/A	integral component of membrane [GO:0016021]
20.	c141404_g1_i1	healthy specific	–	0.21	D0NNS1_PHYIT	voltage-gated Ion Channel superfamily	N/A	small conductance calcium-activated potassium channel activity [GO:0016286]	integral component of membrane [GO:0016021]

Table 1 (continued)

Sl. No.	Transcript ID	Regulation	Log2 fold change	*P*-value	Orthologous ID	Gene Name	GO		
							Biological function	Molecular function	Cellular
21.	c11966_g1_i1	healthy specific	–	0.16	M0SUW6_MUSAM	MATE efflux family	N/A	antiporter activity [GO:0015297]; drug transmembrane transporter activity [GO:0015238]	integral component of membrane [GO:0016021]
22.	c75260_g1_i1	healthy specific	–	0.00	D0EUY7_MEDSA	Ca^{2+} and calmodulin--dependent protein kinase	N/A	ATP binding [GO:0005524]; calcium ion binding [GO:0005509]; protein kinase activity [GO:0004672]	N/A

found to be up-regulated in yellow decline disease affected coconut palms (Nejat et al. 2015).

DEGs related to cell wall fortification

The results obtained in this study revealed *de novo* expression or up-regulation of genes involved in cell wall biogenesis and fortification in diseased palms. Plant cell walls, composed of a network of cellulose and hemicellulose which are embedded in pectin matrix, are an important component in plant-pathogen interactions as they are the first external barriers to prevent pathogen infection. It is evident, therefore, that the integrity of cell wall determines susceptibility of plants to phytopathogens (Lionetti et al. 2012). Pectin methyl esterase (PME), which plays has a crucial role in cell strengthening (Willats et al. 2001), was up-regulated significantly in diseased CGD palms. PME activity plays a significant role in deciding the outcome of plant-pathogen interactions –the enhanced activity of PME makes pectin, present in the plant cell wall, more vulnerable to enzymes produced by phytopathogens (Lionetti et al. 2012). Phytopathogens also break down polymers constituting cell wall into nutritional substrates, which might support growth of the pathogen and subsequent colonization (Cantu et al. 2008; Bellincampi et al. 2014). In addition, pectin demethylesterifcation, catalyzed by PME, renders pectin vulnerable to polygalacturonase hydrolysis (Lionetti et al. 2007). It has been hypothesized that phytoplasma might possess the ability to modify the expression of cell-wall related enzymes, which could augment release of effectors capable of targeting host molecules into host cells and thus facilitate their attack and subsequent multiplication in host cells (Sugio et al. 2011). Upregulation of PME, as observed in the present study, has earlier been reported in citrus in response to Huanglongbing disease (Martinelli et al. 2012), *Paulownia* infected by Witches'–Broom phytoplasma (Mou et al. 2013) and Mexican lime infected with Candidatus *Phytoplasma aurantifolia* (Mardi et al. 2015).

DEGs related to transcription factors

In plants, transcription factors have been recognized for their involvement in regulation and control of plant cellular and developmental functions (McGrath et al. 2005). Transcription factors of AP2/ERF, WRKY and MYB families participate in an elaborate regulation

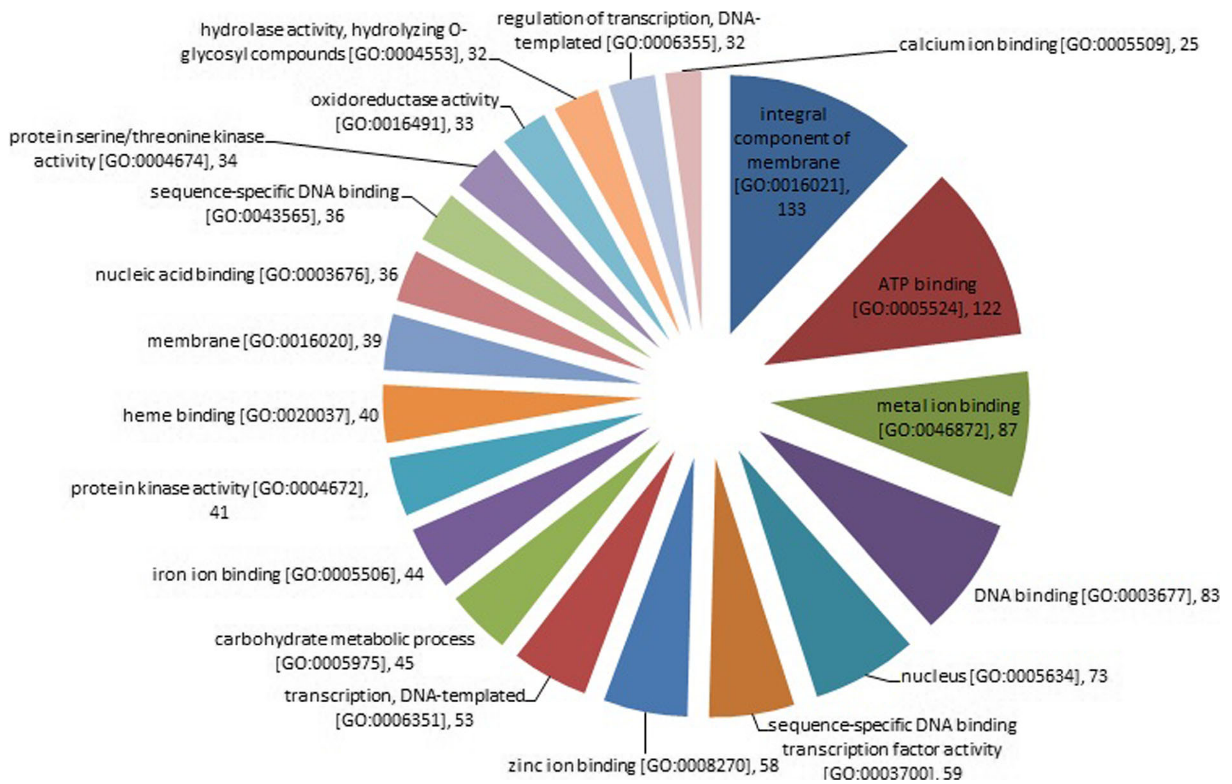

Fig. 5 Top 20 gene ontology (GO) categories harboring specific and differentially expressed transcripts

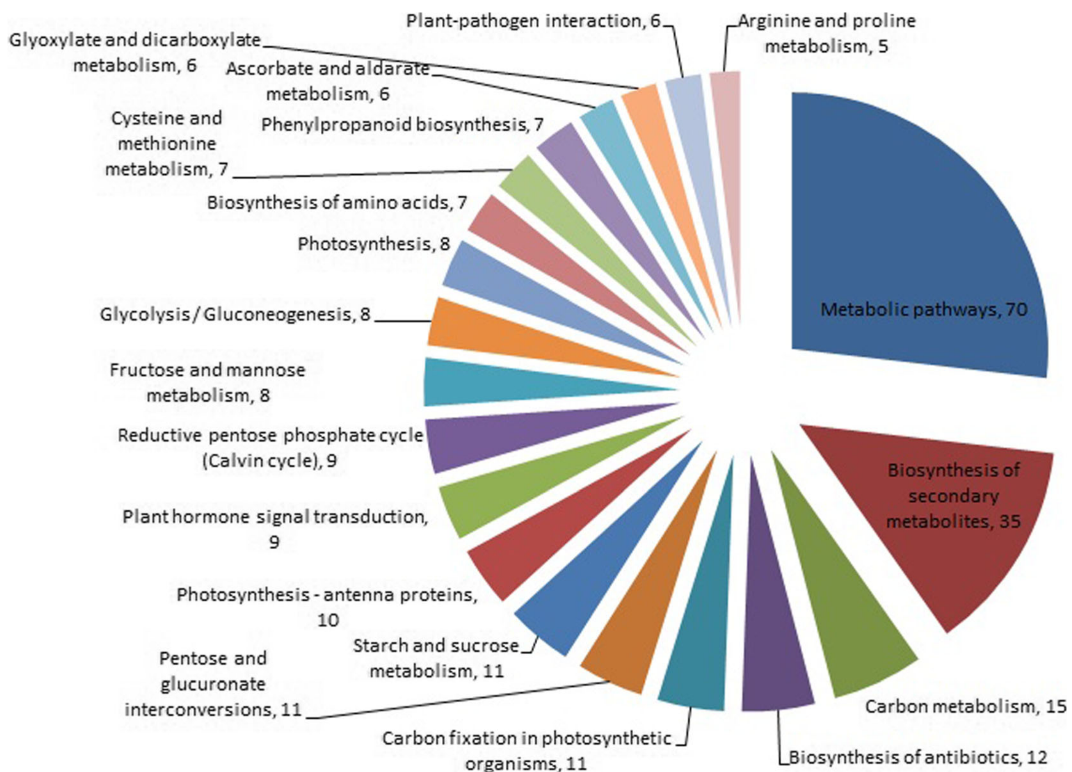

Fig. 6 Top 20 pathways harboring specific and differentially expressed transcripts

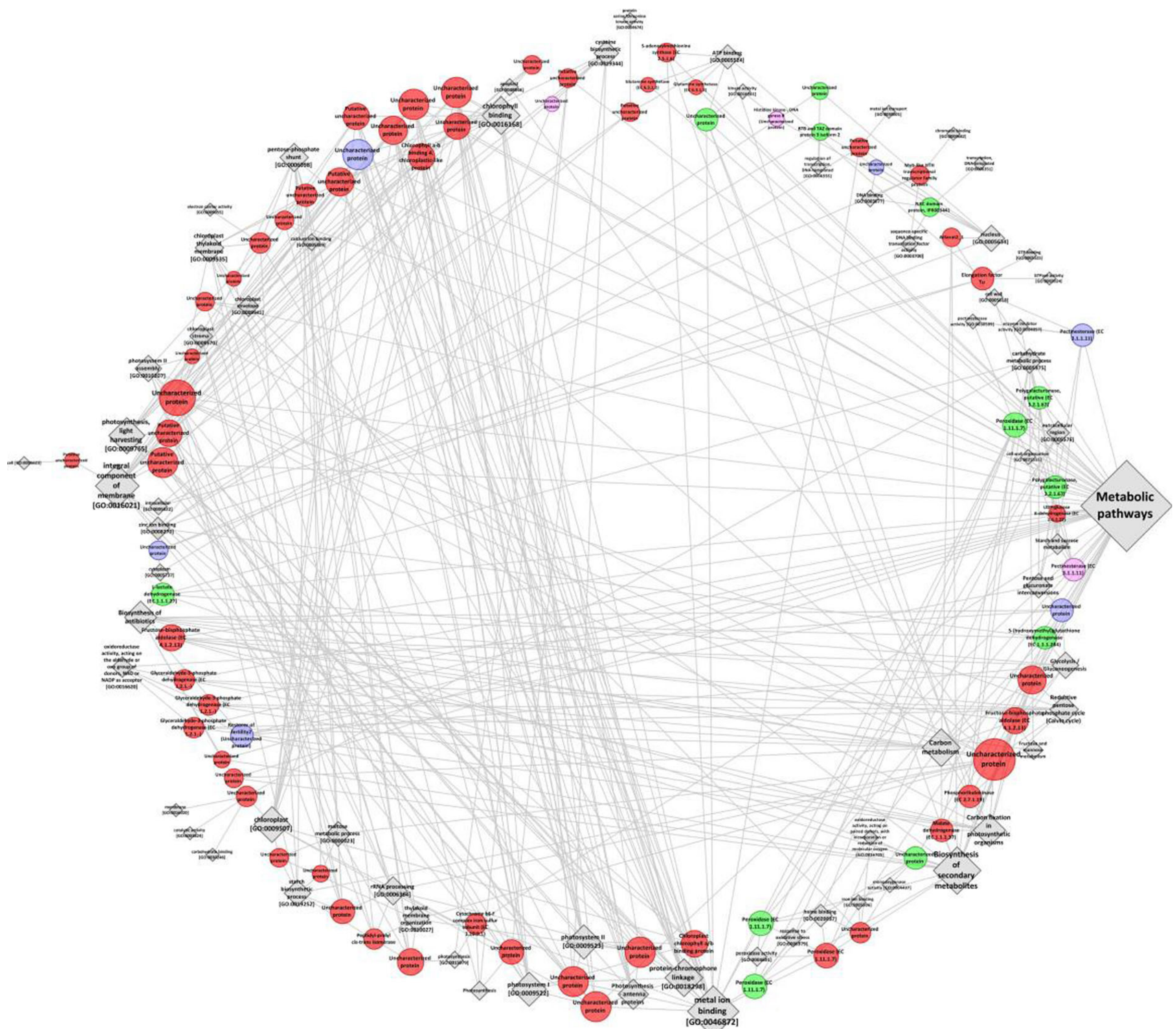

Fig. 7 Gene regulatory network comprising of key genes and GO / Pathways underlying healthy and diseased sample phenotype

network by interacting with target genes; they have been associated with many processes which include activation of defense gene expressions and regulation of phytohormones crosstalk (Atkinson and Urwin 2012; Kissoudis et al. 2014; Seo and Choi 2015). In the present study, a transcript encoding transcription factor RF2a (c29333_g1_i1) was found expressed only in diseased palms. RF2a transcription factors have been reported to be important for the development of rice (Yin et al. 1997) and tobacco (Petruccelli et al. 2001). In rice, two basic leucine zipper (bZIP)-type proteins, RF2a and RF2b, have been reported to interact, both *in vitro* and *in vivo,* with Box II and activate transcription from rice tungro bacilliform virus (RTBV) promoter (Dai et al. 2006). RTBV causes redeployment of vital host transcription machinery to support transcription of the viral promoter in lieu of host genes. Furthermore, the viral promoter may cause a perturbation in the expression of genes that are vital for many processes including plant growth, development and defense, culminating in the manifestation of disease symptoms (Dai et al. 2008).

Another transcription factor MYB (c45098_g2_i1) was found to be specifically expressed in healthy CGD palms. Constituting one of the largest transcription factor family in plants, MYB is known to regulate diverse processes ranging from plant development, differentiation, metabolism to responses to environmental stimuli,

Table 2 List of statistically significant and enriched GO and Pathways with number of genes differentially expressed and specifically expressed

Sl. No.	Gene Ontology / Pathways	Up-regulated	Down-regulated	Disease-specific	Healthy-specific
1.	Metabolic pathways	28	9	1	4
2.	Metal ion binding	16	4	0	1
3.	Biosynthesis of secondary metabolites	11	6	0	2
4.	Integral component of membrane	16	0	0	1
5.	Chlorophyll binding	13	0	0	1
6.	Protein-chromophore linkage	13	0	0	1
7.	Carbon metabolism	12	2	0	0
8.	Photosynthesis, light harvesting	12	0	0	1
9.	Chloroplast	11	0	1	1
10.	Photosystem I	11	0	0	1
11.	Photosystem II	11	0	0	1
12.	Carbon fixation in photosynthetic organisms	10	1	0	0
13.	Photosynthesis - antenna proteins	9	0	0	1

including biotic and abiotic stresses (Ambawat et al. 2013). In *Arabidopsis*, abscisic acid signals mediated by MYB96 have been reported to induce pathogen resistance responses mainly by promoting biosynthesis of SA suggesting the role MYB96 to act as a molecular link mediating ABA-SA crosstalks (Seo and Park 2010).

Transcripts encoding NAC domain protein (c22426_g1_i2, c22426_g1_i1, c44929_g1_i1, c44929_g1_i2 and c44929_g1_i3) were found to be down-regulated in diseased CGD palms. NAC transcriptional factors have been associated with transcriptional remodeling connected with plant immune responses (Nuruzzaman et al. 2013). There are many recent reports citing the role of NAC transcriptional factors as positive regulators of plant defense through the regulated expression of other known defense- and signaling-related genes (Sun et al. 2013; Toth et al. 2016).

In addition to their roles in diverse biological processes, many studies have established the key role mediated by WRKY proteins in both effector-triggered (ETI) and pathogen-associated molecular patterns-triggered (PAMP) immunity (Chi et al. 2013). These transcription factors have the capacity to negatively and positively regulate plant immunity against phytopathogens (Mao et al. 2011). A transcript encoding WRKY DNA-binding protein (c38031_g1_i1) was downregulated in diseased CGD palms. Nejat et al. (2015) had reported significant induction as well as repression of *WRKY* genes in coconut palms infected with yellow decline phytoplasma.

DEGs related to signaling

Receptor-Like kinases (RLKs) appear to play a vital role in signaling, not only during the initial pathogen perception, but also in the ensuing activation of the plant defense response machinery (Afzal et al. 2008). It has been reported that plant RLK perceive external signals through their extracellular domains and subsequently propagate these signals through their intracellular kinase domains (Shiu and Bleecker 2001). A transcript encoding LRR receptor-like serine/threonine-protein kinase (c23220_g1_i2) was specifically expressed in diseased palms. Plant RLKs, which are involved in immunity, function as pattern-recognition receptors which can specifically perceive pathogen-associated molecular patterns (PAMPs) and trigger defense responses, known as PAMP-triggered immunity (PTI), upon binding of their cognate elicitors (Greeff et al. 2012).

Histidine kinase (c138193_g1_i1) was specifically expressed in diseased palms. In plants, histidine-to-aspartate (His-Asp) phosphorelays have been implicated in the control of a range of biological processes, including perception of plant hormones (Osakabe et al. 2013). Phosphorylation of proteins, mediated by protein kinases, is a mechanism utilized for intracellular signaling (Pham et al. 2012). Well defined role has also been attributed to histidine kinases, which function as a part of two-component system, in plant signal transduction (Grefen and Harter 2004).

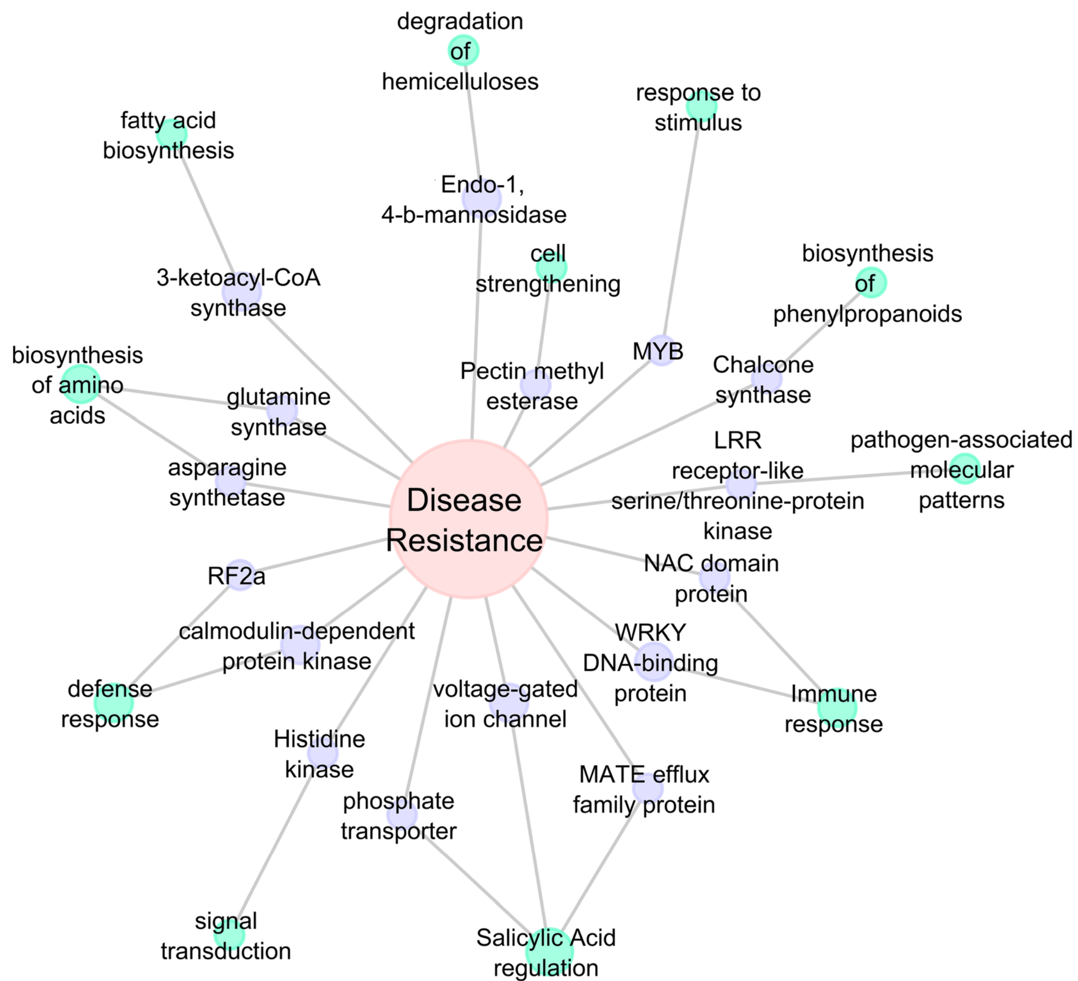

Fig. 8 Model for coconut defense response to root (wilt) disease

Calcium (Ca^{2+}) has been known to activate downstream signaling events leading to perception of defense responses by serving as a second messenger (Lecourieux et al. 2006). Rapid accumulation of Ca^{2+}, in response to phytopathogen attack, has been reported in the cytoplasm paving the way for opening of membrane channels and subsequent signaling leading of activation of plant defense responses (Lecourieux et al. 2006). Ca^{2+} and calmodulin-dependent protein kinase (c75260_g1_i1) was specifically expressed in healthy CGD palms, which possibly points to its role in defense-related signaling cascade in coconut.

DEGs related to transporter families

A transcript encoding phosphate transporter (c61339_g1_i1) was specifically expressed in diseased CGD palms. Members of phosphate transporter family have been reported to be involved in SA regulation (Wang et al. 2011). In *Arabidopsis*, a phosphate transporter *PHT4;1* has been shown to interact with several SA genes and regulate defense responses (Wang et al. 2014). Two transcripts *viz.*, voltage-gated ion channel (c141404_g1_i1) and MATE efflux family protein (c11966_g1_i1) were specifically expressed in healthy palms. Changes in ion fluxes have been reported as one of the early events observed upon recognition of phtyopathogens and therefore are considered as an essential component required for the activation of plant defense machinery. It has been proposed that cyclic nucleotide-gated ion channels (CNGCs) could be involved in modulating these changes in ion fluxes in reponse to pathogen attack (Moeder et al. 2011). The multidrug and toxic compound extrusion (MATE)

transporters, with well defined roles in flavonoid transport, have been identified to play crucial roles in signaling associated with SA-dependent disease resistance in *Arabidopsis* (Nawrath et al. 2002). We speculate that the transporters reported in this study could be involved in defence response to root (wilt) disease in coconut.

A model for coconut defense response to root (wilt) disease

Based on our study on coconut leaf transcriptome, we propose a brief model for the deployment of host defense in coconut against root (wilt) disease (Fig. 8). The host defense is possibly initiated in coconut with the interaction between membrane receptors (LRRs and STKS), and PAMPs or effectors. This interaction triggers downstream signal by the phosphorylation of kinases (histidine kinase) or by bringing changes in calcium-binding proteins (calmodulin) by regulating Ca^{2+} influxes. The phytohormone SA is also recruited into the signaling cascades, and its activity is possibly regulated by the members of transporter families such as phosphate transporter, voltage-gated ion channel and MATE efflux family protein. Together, different signaling cascades orchestrate transcriptional reprogramming with the help of transcription factors WRKY and NAC domain proteins, chiefly activating genes involved in defense metabolites production including phenylpropanoids, which also have a key role in cell wall fortification. The data also indicated the activation of several genes associated with the production of primary metabolites such as carbohydrates, amino acids and lipids and this might have a positive role in the regulation of defense metabolite production.

Conclusions

In this study, RNA-Seq technology was successfully applied for analyzing the global transcriptome profile of healthy and diseased coconut genotypes in response to root (wilt) disease. The results revealed complex interactions between coconut palms and the pathogen which will substantially enhance our understanding of the largely unknown mechanisms that underlie root (wilt) disease of coconut. Based on differential gene expression profiling analysis, 2718 transcripts were observed to be up- or down-regulated in the diseased palms in comparison to healthy ones. Transcripts involved in host energy metabolism, cell wall fortification, signaling, transport and transcription were differentially represented between healthy and diseased palms. We conclude that root (wilt) disease might lead to perturbations in host energy metabolism, transport and transcription, in addition to alterations in cell wall and abnormalities in cell signaling in susceptible genotypes. The data generated will tremendously enhance our understanding of the complex mechanism of resistance to root (wilt) disease. Functional characterization of these transcripts would definitely provide an extensive and crucial overview of the molecular machinery governing host-pathogen interactions in coconut. In addition, further comprehension of disease resistance mechanisms will help coconut researchers to utilize the resistance genes in coconut breeding improvement programs for development of disease resistant varieties.

Acknowledgements The authors wish to thank Indian Council of Agricultural Research (ICAR) for funding.

Author Contributions MKR and AK conceived and designed this research. MKR, KER, RJT and AK conducted the experiments. MKR, KK, KER and BBS analyzed the data. MKR and BBS wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human studies and participants There was no involvement of human participants and/or animals in the present study.

Informed consent The research does not involve informed consent.

References

Abbà, S., Galetto, L., Carle, P., Carrère, S., Delledonne, M., Foissac, X., Palmano, S., Veratti, F., & Marzachì, C. (2014). RNA-Seq profile of flavescence dorée phytoplasma in grapevine. *BMC Genomics, 15*(1), 1088. https://doi.org/10.1186/1471-2164-15-1088.

Afzal, A. J., Wood, A. J., & Lightfoot, D. A. (2008). Plant receptor-like serine threonine kinases: Roles in signaling and plant defense. *Molecular Plant-Microbe Interactions, 21*, 507–517.

Ambawat, S., Sharma, P., Yadav, N. R., & Yadav, R. C. (2013). MYB transcription factor genes as regulators for plant

responses: An overview. *Physiology and Molecular Biology of Plants, 19*, 307–321.

Anders, S., & Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biology, 11*, R106. https://doi.org/10.1186/gb-2010-11-10-r106.

Atkinson, N. J., & Urwin, P. E. (2012). The interaction of plant biotic and abiotic stresses: From genes to the field. *Journal of Experimental Botany, 63*, 3523–3543.

Bankar, K. G., Todur, V. N., Shukla, R. N., & Vasudevan, M. (2015). Ameliorated *de novo* transcriptome assembly using Illumina paired end sequence data with Trinity Assembler. *Genomics Data, 5*, 352–359.

Beliën, T., van Campenhout, S., Robben, J., & Volckaert, G. (2006). Microbial endoxylanases: Effective weapons to breach the plant cell-wall barrier or, rather, triggers of plant defense systems? *Molecular Plant-Microbes Interaction, 19*, 1072–1081.

Bellincampi, D., Cervone, F., & Lionetti, V. (2014). Plant cell wall dynamics and wall-related susceptibility in plant–pathogen interactions. *Frontiers in Plant Science, 5*, 30–37.

Berger, S., Sinha, A. K., & Roitsch, T. (2007). Plant physiology meets phytopathology: Plant primary metabolism and plant–pathogen interactions. *Journal of Experimental Botany, 58*, 4019–4026.

Blacklock, B. J., & Jaworski, J. G. (2006). Substrate specificity of *Arabidopsis* 3-ketoacyl-CoA synthases. *Biochemical and Biophysical Research Communications, 346*, 583–590.

Cantu, D., Vicente, A. R., Labavitch, J. M., Bennett, A. B., & Powell, A. L. (2008). Strangers in the matrix: Plant cell walls and pathogen susceptibility. *Trends in Plant Science, 13*, 610–617.

Chi, Y., Yang, Y., Zhou, Y., Zhou, J., Fan, B., Yu, J. Q., & Chen, Z. (2013). Protein-protein interactions in the regulation of WRKY transcription factors. *Molecular Plant, 6*, 287–300.

Dai, S., Zhang, Z., Bick, J., & Beachy, R. N. (2006). Essential role of the Box II cis element and cognate host factors in regulating the promoter of Rice tungro bacilliform virus. *Journal of General Virology, 87*, 715–722.

Dai, S., Wei, X., Alfonso, A. A., Pei, L., Duque, U. G., Zhang, Z., Babb, G. M., & Beachy, R. N. (2008). Transgenic rice plants that over express transcription factors RF2a and RF2b are tolerant to rice tungro virus replication and disease. *Proceedings of the National Academy of Sciences, 105*, 21012–21016.

Dao, T. T. H., Linthorst, H. J. M., & Verpoorte, R. (2011). Chalcone synthase and its functions in plant resistance. *Phytochemistry Reviews, 10*, 397–412.

De Hoon, M. J. L., Imoto, S., Nolan, J., & Miyano, S. (2004). Open source clustering software. *Bioinformatics, 20*, 1453–1453.

Eynck, C., Séguin-Swartz, G., Clarke, W. E., & Parkin, I. A. P. (2012). Monolignol biosynthesis is associated with resistance to *Sclerotinia sclerotiorum* in *Camelina sativa*. *Molecular Plant Pathology, 13*, 887–899.

Fan, G., Dong, Y., Deng, M., Zhao, Z., Niu, S., & Xu, E. (2014). Plant-pathogen interaction, circadian rhythm, and hormone-related gene expression provide indicators of phytoplasma infection in *Paulownia fortunei*. *International Journal of Molecular Sciences, 15*(12), 23141–23162.

Fan, G., Cao, X., Niu, S., Deng, M., Zhao, Z., & Dong, Y. (2015). Transcriptome, microRNA, and degradome analyses of the gene expression of *Paulownia* with phytoplamsa. *BMC Genomics, 16*(1), 896. https://doi.org/10.1186/s12864-015-2074-3.

Ferrer, J. L., Austin, M. B., Stewart, C., & Noel, J. P. (2008). Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. *Plant Physiology and Biochemistry, 46*, 356–370.

Greeff, C. C., Roux, M. M., Mundy, J. J., & Petersen, M. M. (2012). Receptor-like kinase complexes in plant innate immunity. *Frontiers in Plant Science, 3*, 209. https://doi.org/10.3389/fpls.2012.00209.

Grefen, C., & Harter, K. (2004). Plant two-component systems: Principles, functions, complexity and cross talk. *Planta, 219*, 733–742.

Gurr, G. M., Johnson, A. C., Ash, G. J., Wilson, B. A. L., Ero, M. M., Pilotti, C. A., Dewhurst, C. F., & You, M. S. (2016). Coconut lethal yellowing diseases: A phytoplasma threat to palms of global economic and social significance. *Frontiers in Plant Science, 7*, 1521. https://doi.org/10.3389/fpls.2016.01521.

Henschel, R., Nista, P. M., Lieber, M., Haas, B. J., Wu, L. S., Nista, P. M., Haas, B. J., & Le Duc, R. D. (2012). Trinity RNA-Seq assembler performance optimization. In *Proceedings of the 1st Conference of* the *Extreme Science and Engineering Discovery Environment*: *Bridging From the Extreme to the Campus and Beyond* (pp. 1–8). ACM.

Huang, Y. Y., Lee, C. P., Fu, J. L., Chang, B. C., Matzke, A. J., & Matzke, M. (2014). *De novo* transcriptome sequence assembly from coconut leaves and seeds with a focus on factors involved in RNA-directed DNA methylation. *G3: Genes Genomes Genetics, 4*, 2147–2157.

Jacob, P. M., Nair, R. V., & Rawther, T. S. S. (1998). Varietal resistance. In K. U. K. Nampoothiri & P. K. Koshy (Eds.), *Coconut Root (wilt) Disease* (pp. 97–104). Kasaragod: CPCRI.

Kangasjärvi, S., Neukermans, J., Li, S., Aro, E. M., & Noctor, G. (2012). Photosynthesis, photorespiration, and light signalling in defence responses. *Journal of Experimental Botany, 63*, 1619–1636.

Kissoudis, C., van de Wiel, C., Visser, R. G. F., & van der Linden, G. (2014). Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk. *Frontiers in Plant Science, 5*, 207. https://doi.org/10.3389/fpls.2014.00207.

Koshy, P. K. (1999). Root (wilt) disease of coconut. *Indian Phytopathology, 52*, 335–353.

Kostyn, K., Czemplik, M., Kulma, A., Bortniczuk, M., Skała, J., & Szopa, J. (2012). Genes of phenylpropanoid pathway are activated in early response to *Fusarium* attack in flax plants. *Plant Science, 190*, 103–115.

Lecourieux, D., Raneva, R., & Pugin, A. (2006). Calcium in plant defence-signalling pathways. *New Phytologist, 171*, 249–269.

Lionetti, V., Raiola, A., Camardella, L., Giovane, A., Obel, N., Pauly, M., Favaron, F., Cervone, F., & Bellincampi, D. (2007). Overexpression of pectin methylesterase inhibitors in *Arabidopsis* restricts fungal infection by *Botrytis cinerea*. *Plant Physiology, 143*, 1871–1880.

Lionetti, V., Cervone, F., & Bellincampi, D. (2012). Methyl esterification of pectin plays a role during plant–pathogen interactions and affects plant resistance to diseases. *Journal of Plant Physiology, 169*, 1623–1630.

Liu, R., Dong, Y., Fan, G., Zhao, Z., Deng, M., Cao, X., & Niu, S. (2013). Discovery of genes related to witches broom disease

in *Paulownia tomentosa* × *Paulownia fortunei* by a *de novo* assembled transcriptome. *PLoS One, 8*(11), e80238. https://doi.org/10.1371/journal.pone.0080238.

Liu, L. Y., Tseng, H. I., Lin, C. P., Lin, Y. Y., Huang, Y. H., Huang, C. K., Chang, T. H., & Lin, S. S. (2014). High-throughput transcriptome analysis of the leafy flower transition of *Catharanthusroseus* induced by peanut witches'-broom phytoplasma infection. *Plant Cell Physiology, 55*(5), 942–957.

Manimekalai, R., Soumya, V. P., Sathish Kumar, R., Selvarajan, R., Reddy, K., Thomas, G. V., Sasikala, M., Rajeev, G., & Baranwal, V. K. (2010). Molecular detection of 16SrXI group phytoplasma associated with root (wilt) disease of coconut (*Cocos nucifera* L.) in India. *Plant Disease, 94*, 636–636.

Mao, G., Meng, X., Liu, Y., Zheng, Z., Chen, Z., & Zhang, S. (2011). Phosphorylation of a WRKY transcription factor by two pathogen-responsive MAPKs drives phytoalexin biosynthesis in *Arabidopsis*. *Plant Cell, 23*, 1639–1653.

Mardi, M., Karimi Farsad, L., Gharechahi, J., & Salekdeh, G. H. (2015). In-depth transcriptome sequencing of Mexican lime trees infected with Candidatus *Phytoplasma aurantifolia*. *PLoS One, 10*(7), e0130425. https://doi.org/10.1371/journal.pone.0130425.

Martinelli, F., Uratsu, S. L., Albrecht, U., Reagan, R. L., Phu, M. L., Britton, M., Buffalo, V., Fass, J., Leicht, E., Zhao, W., & Lin, D. (2012). Transcriptome profiling of citrus fruit response to Huanglongbing disease. *PLoS One, 7*(5), e38039. https://doi.org/10.1371/journal.pone.0038039.

McGrath, K. C., Dombrecht, B., Manners, J. M., Schenk, P. M., Edgar, C. I., Maclean, D. J., Scheible, W. R., Udvardi, M. K., & Kazan, K. (2005). Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of *Arabidopsis* transcription factor gene expression. *Plant Physiology, 139*, 949–959.

Moeder, W., Urquhart, W., Ung, H., & Yoshioka, K. (2011). The role of cyclic nucleotide-gated ion channels in plant immunity. *Molecular Plant, 4*, 442–452.

Mou, H. Q., Lu, J., Zhu, S. F., Lin, C. L., Tian, G. Z., Xu, X., & Zhao, W. J. (2013). Transcriptomic analysis of *Paulownia* infected by *Paulownia* witches'-broom Phytoplasma. *PLoS One, 8*, e77217. https://doi.org/10.1371/journal.pone.0077217.

Nagalakshmi, U., Waern, K., & Snyder, M. (2010). RNA-Seq: A method for comprehensive transcriptome analysis. *Current Protocols in Molecular Biology*. https://doi.org/10.1002/0471142727.mb0411s89.

Nair, M. K., Koshy, P. K., Jacob, P. M., Nair, R. V., Bhaskara Rao, E. V. V., Nampoothiri, K. U. K., & Iyer, R. D. (1996). A root (wilt) disease resistant coconut hybrid and strategy for resistance breeding. *Indian Coconut Journal, 27*, 2–5.

Nair, R. V., Jacob, P. M., & Ajithkumar, R. (2004). Screening of coconut varieties against root (wilt) disease. *Journal of Plantation Crops, 32*, 50–51.

Nambiar, P. T. N., & Pillai, N. G. (1985). A simplified method of indexing root (wilt) affected coconut palms. *Journal of Plantation Crops, 13*, 35–37.

Nawrath, C., Heck, S., Parinthawong, N., & Métraux, J. P. (2002). EDS5, an essential component of salicylic acid–dependent signaling for disease resistance in *Arabidopsis*, is a member of the MATE transporter family. *The Plant Cell, 14*, 275–286.

Nejat, N., Sijam, K., Abdullah, S. N. A., Vadamalai, G., & Dickinson, M. (2009). Molecular characterization of a phytoplasma associated with coconut yellow decline (CYD) in Malaysia. *American Journal of Applied Sciences, 6*, 1331–1340.

Nejat, N., Cahill, D. M., Vadamalai, G., Ziemann, M., Rookes, J., & Naderali, N. (2015). Transcriptomics-based analysis using RNA-Seq of the coconut (*Cocos nucifera*) leaf in response to yellow decline phytoplasma infection. *Molecular Genetics and Genomics, 290*, 1899–1910.

Nuruzzaman, M., Sharoni, A. M., & Kikuchi, S. (2013). Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. *Frontiers in Microbiology, 4*, 248. https://doi.org/10.3389/fmicb.2013.00248.

Osakabe, Y., Yamaguchi-Shinozaki, K., Shinozaki, K., & Tran, L. S. (2013). Sensing the environment: Key roles of membrane-localized kinases in plant perception and response to abiotic stress. *Journal of Experimental Botany, 64*, 445–458.

Ozsolak, F., & Milos, P. M. (2011). RNA sequencing: Advances, challenges and opportunities. *Nature Reviews Genetics, 12*, 87–98.

Perera, S. A. C. N., Herath, H. M. N. B., Wijesekera, H. T. R., Subhathma, W. G. R., & Weerakkody, W. A. T. L. (2015). Evaluation of coconut germplasm in Weligama and Matara area of the Southern Province of Sri Lanka for resistance to Weligama coconut leaf wilt disease. *Cocos, 21*, 15–20.

Petruccelli, S., Dai, S., Carcamo, R., Yin, Y., Chen, S., & Beachy, R. N. (2001). Transcription factor RF2a alters expression of the rice tungro bacilliform virus promoter in transgenic tobacco plants. *Proceedings of the National Academy of Sciences, 98*, 7635–7640.

Pfaffl, M. W., Horgan, G. W., & Dempfle, L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research, 30*, e36.

Pham, J., Liu, J., Bennett, M. H., & Mansfield, J. W. (2012). *Arabidopsis* histidine kinase 5 regulates salt sensitivity and resistance against bacterial and fungal infection. *New Phytologist, 194*, 168–180.

Quaicoe, R. N., Dery, S. K., Philippe, R., Baudouin, L., Nipah, J. O., Nkansah-Poku, J., Arthur, R., Dare, D., Yankey, E. N., Pilet, F., & Dollet, M. (2009). Resistance screening trials on coconut varieties to Cape Saint Paul Wilt Disease in Ghana. *Oléagineux, 16*, 132–136.

Raffaele, S., Leger, A., & Roby, D. (2009). Very long chain fatty acid and lipid signaling in the response of plants to pathogens. *Plant Signaling & Behavior, 4*, 94–99.

Rajesh, M. K., Rachana, K. E., Fayas, T. P., Babu, M., Kiran, A. G., & Karun, A. (2014). Selection and validation of reference genes for quantitative gene expression studies by real-time PCR in coconut. In K. Muralidharan, M. K. Rajesh, K. S. Muralikrishna, J. Vijayan, & S. Jayasekhar (Eds.), *Book of Abstracts of National Seminar on Sustainability of Coconut, Arecanut and Cocoa Farming-Technological Advances and Way Forward* (p. 34). Kasaragod: ICAR-CPCRI.

Rajesh, M. K., Fayas, T. P., Naganeeswaran, S., Rachana, K. E., Bhavyashree, U., Sajini, K. K., & Karun, A. (2016). *De novo* assembly and characterization of global transcriptome of coconut palm (*Cocos nucifera* L.) embryogenic calli using Illumina paired-end sequencing. *Protoplasma, 253*, 913–928.

Rojas, C. M., Senthil-Kumar, M., Tzin, V., & Mysore, K. S. (2014). Regulation of primary plant metabolism during

plant-pathogen interactions and its contribution to plant defense. *Frontiers in Plant Science, 5*, 17. https://doi.org/10.3389/fpls.2014.00017.

Saensuk, C., Wanchana, S., Choowongkomon, K., Wongpornchai, S., Kraithong, T., Imsabai, W., Chaichoompu, E., Ruanjaichon, V., Toojinda, T., Vanavichit, A., & Arikit, S. (2016). *De novo* transcriptome assembly and identification of the gene conferring a "pandan-like" aroma in coconut (*Cocos nucifera* L.) *Plant Science, 252*, 324–334.

Saldanha, A. J. (2004). Java Tree view-extensible visualization of microarray data. *Bioinformatics, 20*, 3246–3248.

Sanchez, F. I. J. (2008). *Polyketide synthases in Cannabis sativa* L. PhD thesis, Pharmacognosy Department, Section of Metabolomics, Institute of Biology, Faculty of Science, Leiden University.

Sánchez-Rangel, D., Rivas-San Vicente, M., de la Torre-Hernández, M. E., Nájera-Martínez, M., & Plasencia, J. (2014). Deciphering the link between salicylic acid signaling and sphingolipid metabolism. *Frontiers in Plant Science, 6*, 125. https://doi.org/10.3389/fpls.2015.00125.

Sasikala, M., Rajeev, G., Prakash, V. R., & Amith, S. (2010). Modified protocol of ELISA for rapid detection of coconut root (wilt) disease. *Journal of Plantation Crops, 38*, 16–19.

Scheideler, M., Schlaich, N. L., Fellenberg, K., Beissbarth, T., Hauser, N. C., Vingron, M., Slusarenko, A. J., & Hoheisel, J. D. (2002). Monitoring the switch from housekeeping to pathogen defense metabolism in *Arabidopsis thaliana* using cDNA arrays. *Journal of Biological Chemistry, 277*, 10555–10561.

Seifi, H. S., Van Bockhaven, J., Angenon, G., & Höfte, M. (2013). Glutamate metabolism in plant disease and defense: Friend or foe? *Molecular Plant-Microbe Interactions, 26*, 475–485.

Seifi, H. S., De Vleesschauwer, D., Aziz, A., & Höfte, M. (2014). Modulating plant primary amino acid metabolism as a necrotrophic virulence strategy: The immune-regulatory role of asparagine synthetase in *Botrytis cinerea*-tomato interaction. *Plant Signaling & Behavior, 9*, e27995.

Seo, E., & Choi, D. (2015). Functional studies of transcription factors involved in plant defenses in the genomics era. *Briefings in Functional Genomics, 14*, 260–267.

Seo, P. J., & Park, C. M. (2010). MYB96-mediated abscisic acid signals induce pathogen resistance response by promoting salicylic acid biosynthesis in *Arabidopsis*. *New Phytologist, 186*, 471–483.

Shiu, S. H., & Bleecker, A. B. (2001). Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases. *Proceedings of the National Academy of Sciences, 98*, 10763–10768.

Siewert, C., Luge, T., Duduk, B., Seemüller, E., Büttner, C., Sauer, S., & Kube, M. (2014). Analysis of expressed genes of the bacterium 'Candidatus phytoplasma Mali' highlights key features of virulence and metabolism. *PLoS One, 9*(4), e94391. https://doi.org/10.1371/journal.pone.0094391.

Singh, P. K., Akram, M., Vajpeyi, M., Srivastava, R. L., Kumar, K., & Naresh, R. (2007). Screening and development of resistant sesame varieties against phytoplasm. *Bulletin of Insectology, 60*, 303–304.

Solomon, J. J., Govindankutty, M. P., & Nienhaus, F. (1983). Association of mycoplasma-like organisms with the coconut root (wilt) disease in India. *ZeitschriftfuerPflanzenkrankheiten und Pflanzenschutz, 90*, 295–297.

Sugio, A., MacLean, A. M., Grieve, V. M., & Hogenhout, S. A. (2011). Phytoplasma protein effector SAP11 enhances insect vector reproduction by manipulating plant development and defense hormone biosynthesis. *Proceedings of the National Academy of Sciences, 108*, E1254–E1263.

Sun, L., Zhang, H., & Li, D. (2013). Functions of rice NAC transcriptional factors, ONAC122 and ONAC131, in defense responses against *Magnaporthe grisea*. *Plant Molecular Biology, 81*, 41–56.

Thomas, R. J., Rajesh, M. K., Jacob, P. M., Jose, M., & Nair, R. V. (2015). Studies on genetic uniformity of Chowghat Green Dwarf and Malayan Green Dwarf varieties of coconut using molecular and morphometric methods. *Journal of Plantation Crops, 43*, 89–96.

Toth, Z., Winterhagen, P., Kalapos, B., Su, Y., Kovacs, L., & Kiss, E. (2016). Expression of a grapevine NAC transcription factor gene is induced in response to powdery mildew colonization in salicylic acid-independent manner. *Scientific Reports, 6*, 30825. https://doi.org/10.1038/srep30825.

Vogt, T. (2010). Phenylpropanoid biosynthesis. *Molecular Plant, 3*, 2–20.

Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics, 10*, 57–63.

Wang, G. Y., Shi, J. L., Ng, G., Battle, S. L., Zhang, C., & Lu, H. (2011). Circadian clock-regulated phosphate transporter PHT4;1 plays an important role in *Arabidopsis* defense. *Molecular Plant, 4*, 516–526.

Wang, G., Zhang, C., Battle, S., & Lu, H. (2014). The phosphate transporter PHT4; 1 is a salicylic acid regulator likely controlled by the circadian clock protein CCA1. *Frontiers in Plant Science, 5*, 701. https://doi.org/10.3389/fpls.2014.00701.

Wang, Y., Zhou, L., Yu, X., Stover, E., Luo, F., & Duan, Y. (2016). Transcriptome profiling of Huanglongbing (HLB) tolerant and susceptible citrus plants reveals the role of basal resistance in HLB tolerance. *Frontiers in Plant Science*. https://doi.org/10.3389/fpls.2016.00933.

Ward, J. L., Forcat, S., Beckmann, M., Bennett, M., Miller, S. J., Baker, J. M., Hawkins, N. D., Vermeer, C. P., Lu, C., Lin, W., & Truman, W. M. (2010). The metabolic transition during disease following infection of *Arabidopsis thaliana* by *Pseudomonas syringae* pv. tomato. *Plant Journal, 63*, 443–457.

Willats, W. G., McCartney, L., Mackie, W., & Knox, J. P. (2001). Pectin: Cell biology and prospects for functional analysis. *Plant Molecular Biology, 47*, 9–27.

Yin, Y., Chen, L., & Beachy, R. (1997). Promoter elements required for phloem-specific gene expression from the RTBV promoter in rice. *Plant Journal, 12*, 1179–1188.

Zeier, J. (2013). New insights into the regulation of plant immunity by amino acid metabolic pathways. *Plant, Cell and Environment, 36*, 2085–2103.

Zhang, Q., & Xiao, S. (2015). Lipids in salicylic acid-mediated defense in plants: Focusing on the roles of phosphatidic acid and phosphatidylinositol 4-phosphate. *Frontiers in Plant Science, 6*, 387. https://doi.org/10.3389/fpls.2015.00387.

Zhang, S. H., Yang, Q., & Ma, R. C. (2007). *Erwinia carotovora* ssp. *carotovora* infection induced "defense lignin" accumulation and lignin biosynthetic gene expression in Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*). *Journal of Integrative Plant Biology, 49*, 993–1002.