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COMPARATIVE TRANSCRIPTOME ANALYSIS TO DETERMINE DIFFERENTIALLY EXPRESSED GENES RESPONSIBLE TOWARDS GROWTH OF METROXYLON SAGU

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Abstract: Sago palm (*Metroxylon sagu* Rottb.) is one of the most important cash crops grown abundantly in Mukah, Sarawak, Malaysia. The palm's versatility and high starch content led the Sarawak government to introduce large scale commercial sago estate plantations in efforts to boost the local economy. However, several challenges were encountered, including stunted growth (non-trunking) despite the plants reaching maturity. In spite of many researches to address this, information on the molecular mechanisms of sago palms is relatively low. This study compares the transcriptome datasets expressed in trunking sago palms of different localities. The RNA extracted was subjected to RNA sequencing. Raw data was processed with *fastp,* generating 85.61 Gb clean data, averaging 7 Gb data from each sample. The clean reads were subjected to assembly and mapping using Salmon. Expression count tables were used for DEGs identification using idep.96 webserver. A total of 718 DEGs were commonly expressed in all datasets. Enrichment analysis revealed gene products responsible for maintaining cell stability were enriched in GO, whereas the MAPK and ribosome pathways were significantly enriched in KEGG. The data obtained from this study can be considered for further analysis and understanding, especially toward the genes responsible for normal development in sago palms.

Keywords: *Metroxylon sagu*, non-trunking, transcriptomics, trunking.

Introduction

The global population is predicted to exceed 9.8 billion by 2050. This means an increase in food demand (Ghosh *et al.*, 2024). The world is still struggling to meet the demand for agricultural products, and it is uncertain if the world can meet this challenging target. Food and Agricultural Organization of the United Nations (FAOSTAT) reported a stagnant decline in the annual productivity of major cereal crops since 1987 due to land and water scarcity as well as the negative impacts of climate change (Konuma, 2018). This situation has sparked interest in exploring neglected crop species that can grow on underutilised lands, tolerate environmental stresses, and produce substantial quantities of food or starch in battling food scarcity and hunger, maintaining the traditional landscape, and providing economic development to the rural areas (Konuma, 2018).

The sago palm (*Metroxylon sagu* Rottb.) is a monocot halophytic starch-producing plant that grows in the hot, humid tropics of Indonesia, Malaysia, Philippines, Thailand, Papua New Guinea, and Oceanian Islands (Flach, 1997). The palm possesses a remarkable starch content as it can yield up to 25/MT/ha/year surpassing other significant starch-producing crops such as rice (6 MT/ha/year), maize (5.5 MT/ha/year), wheat (5 MT/ha/year), and potato (2.5 MT/ha/year) (Ishizaki, 1997; Ahmad *et al*., 2022). The sago starch is often sought after in food preparation owing to their better gelatinisation

behaviour and amylose content (Hirao *et al.*, 2018). The sago starch is also included in ethanol production, biodegradable plastic products, and animal feed manufacture (Singhal *et al*., 2008; Ehara, 2009). Therefore, owing to the high productivity and starch quality as well as the plant's versatility to thrive in adverse environments, the Sarawak government decided to initiate commercial sago palm plantations to boost its economic growth (Mohamad Naim *et al.*, 2016). However, stunted or non-trunking (no trunk development) sago palms were observed in sago palm plantations despite reaching the maturity period of over 10 years post cultivation (Hussain *et al*., 2020b; Hussain *et al*., 2022; Yan *et al*., 2022). This condition eliminates the economic value of the affected palms, resulting in a decline in sago starch productivity per hectare of land, and subsequently, instability in sago cultivation .

Previous research on soil physicochemical and foliar analyses reported nutrient deficiencies in Non-Trunking (NT) sago palm. A study found starch accumulation in sago palm grown in acid sulfate soil is low compared with sago palm grown in mineral soil (Nozaki *et al.*, 2004). Acclimation processes experienced by the plants to overcome a multitude of environmental stresses allow them to survive but often result in reduced growth of the affected plant (Tan *et al.*, 2023). Proteomics studies which examine molecular activities, have shown that NT palms significantly express genes linked to stress factors (Hussain *et al*., 2020b; Hussain *et al*., 2022). With the current knowledge and the availability of dataset related to NT sago palm, it is also crucial to determine the genes that are commonly expressed on trunking sago palms to uncover genetic mechanisms related to their growth.

This is a comparative analysis of trunking sago palms from different sago plantations vis a vis the NT ones. Leaf samples were collected from trunking sago palms of several semi-wild sago plantations, namely Dalat Sago Plantation, Sungai Talau Research Station, and Paya Paloh Sago Plantation, referred to as Dalat, Sg. Talau, and PPaloh, respectively. Semi-wild plantations refers to an area where the trees are cultivated with minimum maintenance and without proper treatments such as fertiliser applications, occasional weeding, and land clearing (Jong, 1995). These sago palm plantations were selected to study the genes that are stably expressed in trunking sago palms grown on shallow peat soil (Dalat) and mineral soils (PPaloh and Sg. Talau). RNA-sequencing was selected to analyse Differentially Expressed Genes (DEGs). The study aims to understand these regulated genes and pathways in the trunking sago palms from different localities against the non-trunking sago palms. It hopes to shed light on the molecular mechanisms that support the normal (trunking) development of sago palms.

Materials and Methods

Plant Material

The sago palm leaves of the third frond from the bole of *Pelawei Manit* (8 to 12 years) were used for this experiment. The palms were felled to obtain the leaf samples. The collection sites were Dalat and Sungai Talau of Mukah division and Paya Paloh of Kota Samarahan division, Sarawak. Global positioning system (GPS) of each sample can be accessed from figshare repository (https://doi.org/10.6084/m9.figshare.25991932.v1). Three biological replications were collected for each site. Upon collection, the leaf was cleaned with 70% ethanol, rolled into 50 ml Falcon tube labelled accordingly, and snap-freeze in liquid nitrogen for transportation. The samples were stored at -80°C for long-term storage until use. Figure 1 shows the trunking sago palms on the field.

RNA Extraction, Illumina Sequencing, and Transcriptome Assembly

Total RNA was isolated from M. sagu leaves according to (Yan *et al.*, 2022) using CTAB method with three technical replicates of each sample. The tissues were ground to a fine powder in pre-cooled mortars. An additional isopropanol cleanup was conducted to further remove

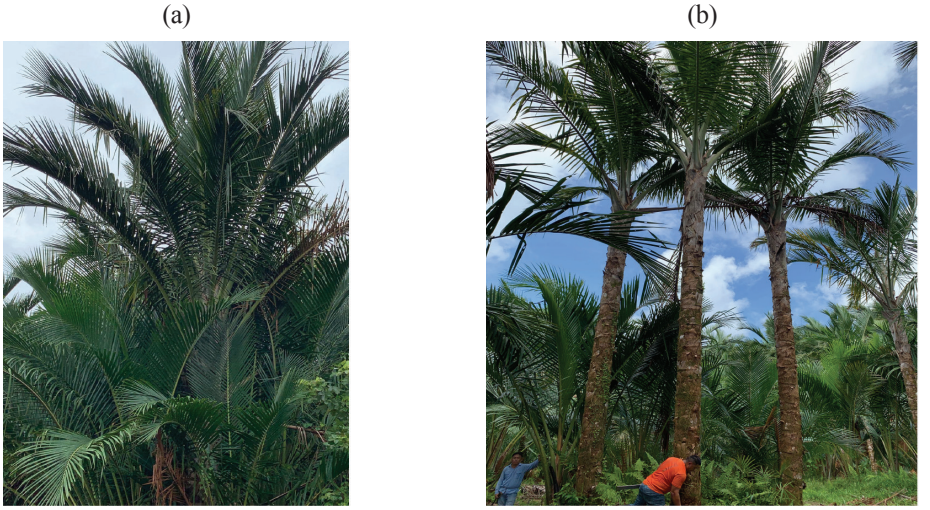

Figure 1: The trunking sago palm on the field. (a) Sago palm from across the road and (b) selected sago palm for leaf collection

contaminants and improve the quality of the total RNA. Total RNA was qualified and quantified using a NanoDrop instrument (DeNovix DS-11, USA) and Agilent 2100 Bioanalyzer (Thermo Fisher Scientific, Waltham, MA, USA). The samples were sequenced using a paired-end strategy on the Illumina NovaSeq 6000 sequencing platform at 1st BASE sequencing facility (Singapore). The raw reads obtained from sequencing were processed using an all-in-one FASTQ preprocessor, *fastp* (Chen *et al.*, 2018) to obtain high-quality reads. The resultant high-quality reads of each sample underwent transcriptome mapping using Salmon quasi-mapper (COMBINE lab).

Differentially Expressed Genes (DEGs) Analysis

The read counts for transcripts were used as input data for differential expression using a web application, iDEP.96 (integrated Differential Expression and Pathway analysis) (Ge *et al.*, 2018). Analysis for Differentially Expressed Genes (DEGs) of all locations (Dalat, Sg. Talau and PPaloh) were compared with control, Non-Trunking (NT) with three biological replications. The DEGs of the three comparison sets (Dalat vs. NT, Sg. Talau vs. NT, PPaloh vs. NT) were identified using DESeq2 software following threshold criteria: False Discovery Rate (FDR) < 0.05 and fold-change > 2. The non-trunking data were obtained from NCBI Bioproject: PRJNA781491 (accession: SAMN23282501, SAMN23282500, SAMN23282499).

Functional Annotation and Enrichment Analysis

The assembled transcripts' gene functions were annotated using a fast annotation tool for novel sequences; EggNOG (Evolutionary Gene Genealogy Non-Supervised Orthologous Group)-mapper database (http://eggnog5.embl.de). Subsequently, the highly expressed genes obtained from differential expression analysis were subjected to Gene Ontology (GO) enrichment analysis and Kyoto Encylopedia of Genes and Genomes (KEGG) enrichment analysis using stand-alone software, TBtools (a Toolkit for Biologists integrating various biological data-handling tools) (Chen *et al.*, 2020).

Identification of Transcription Factors

A homology search of a transcription factor with Arabidopsis genes was performed using plant transcription factor database (PTFB; http://planttfdb.gao-lab.org/).

Results

Quality Assessment and Repeat Correlation Analysis of RNA-Seq Data

Three biological replicates of all samples (12 samples in total) were analysed. A total of 85.61 Gb of clean data were obtained after data filtering, with each sample yielding an average of 7 Gb of data (Table S1). The Phred-scale based quality score "Q" represents the likelihood of an incorrect base-call once every 1,000 bases equivalent to a precision rate of 99.9% was scaled at 30 (Belay *et al.*, 2024). The Phred score (Q30) was found to be more than 90% and the Guanine-Cytosine (GC) content was consistently between 48% to 50%, suggesting high-quality sequencing and meeting the requirements for subsequent data analysis (Luo *et al.*, 2024). The raw reads obtained from RNA sequencing were submitted to the NCBI Sequence Read Archive (SRA) database under Bioproject: PRJNA922330. In order to standardise the expression level of each transcript, Count Per Million (CPM), which filters based on sequencing depth instead of gene length was employed to filter the genes that are not expressed or extremely low in any samples (Giannoukakos *et al.*, 2024). Pearson's correlation coefficients of gene expression levels between the three biological replicates of different samples are between 0.8 and 1, indicating a high degree of homogeneity of genes within each sample and their respective gene expression levels are similar. Any correlation coefficient value lower than 0.8 show low correlation in their samples and respective gene expression levels and vice versa (Sun *et al.*, 2024). The Principal Component Analysis (PCA) further demonstrated the sample replication differentiation was evident as the biological samples were clustered together (Figure 1).

Analysis of DEGs in Different Comparison Groups

Pairwise differential expression analysis was carried out using the negative binomial generalised linear model with shrinkage estimators for dispersion and fold change known as DESeq2 method to examine the DEGs between the three comparative sets; Dalat_NT, Sg. Talau_NT, and PPaloh_NT, following threshold criteria of FDR < 0.05 and log2 fold-change ≥ 2. DEseq2 was preferable as it has sensitivity and precision and control in false positive rate (Love *et al.*, 2014). Under these criteria, 1,022, 836, and 1,522 genes exhibited significant upregulation whereas 1,125, 693, and 834 genes exhibited significant downregulation between Sg. Talau_NT, PPaloh_NT, and Dalat_NT, respectively (Figure 2). The Venn diagram identified a total of 718 common DEGs from the three comparative datasets comprising 402 up- and 315 down-regulated DEGs (Figure 3).

Functional Characterisation of DEGs

To gain functional information on the DEGs, Gene Ontology (GO) classification analysis was performed on the DEGs to classify them based on their functions in Biological Processes (BP), Cellular Component (CC), and Molecular Function (MF) (Figure 4). The GO analyses of both upregulated and downregulated DEGs showed most genes were involved in the biological processes category, followed by the molecular function and cellular component categories (Table S2). Based on the GO analyses, gene products relating to cell stability maintenance were enriched as indicated in the biological process. Leaf senescence (GO: 0010150), intracellular chemical homeostasis (GO: 0055082), and cellular homeostasis (GO: 0019725) were significantly enriched for upregulated DEGs. The downregulated DEGs GO found gene products related to sulphur deficiency and fatty acid derivatives participating in plant defence significantly downregulated as denoted by sulphur amino acid biosynthesis (GO: 0000097), fatty acid derivative metabolic process (GO: 1901568), sulphur amino acid

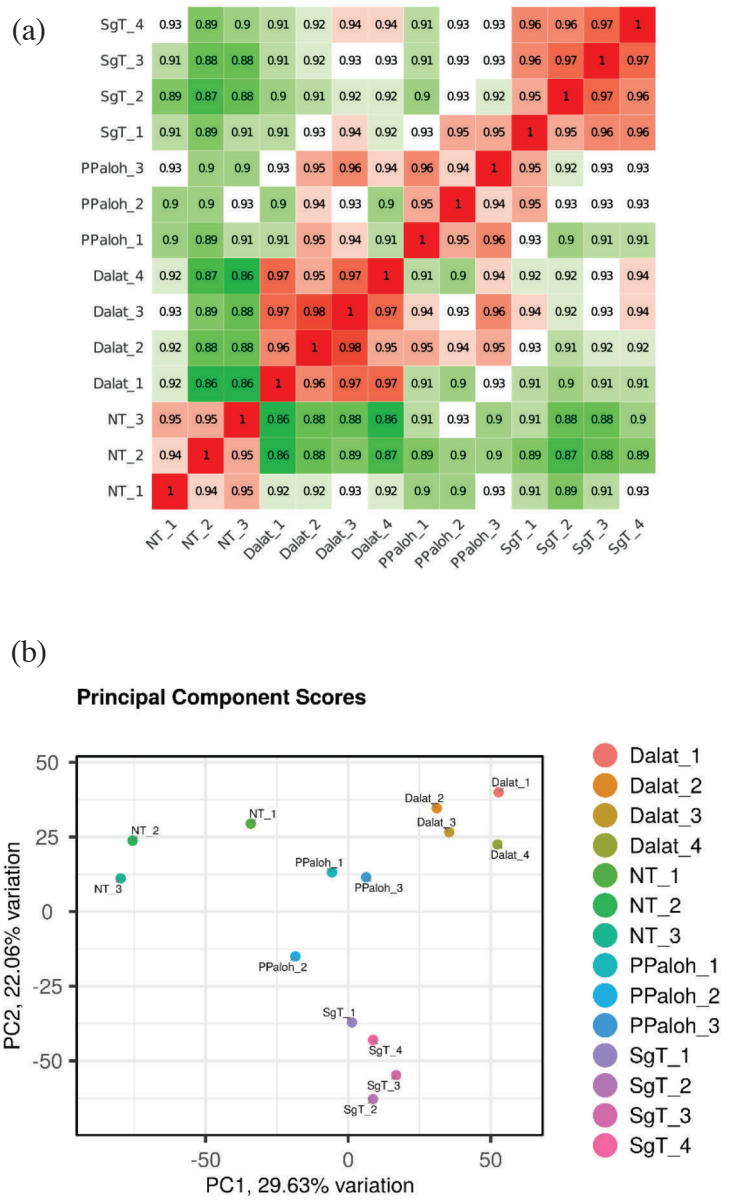

Figure 2: Correlation between transcriptomes samples. (a) Pearson correlation coefficient between three biological replicates of transcriptome samples and (b) PCA plot showing clustering of transcriptome samples

metabolic process (GO: 0006790), and cellular response to toxic substance (GO: 0009636). In the cellular component category, the dominantly enriched GO terms for upregulated DEGs were the ribonucleoprotein complex (GO: 1990904), nucleolus (GO: 0005730), and nuclear lumen (GO: 0031981) whereas the microbody (GO: 0042579), peroxisome (GO: 0005777), and ribosome (GO: 0005840) were significantly enriched GO terms for downregulated DEGs.

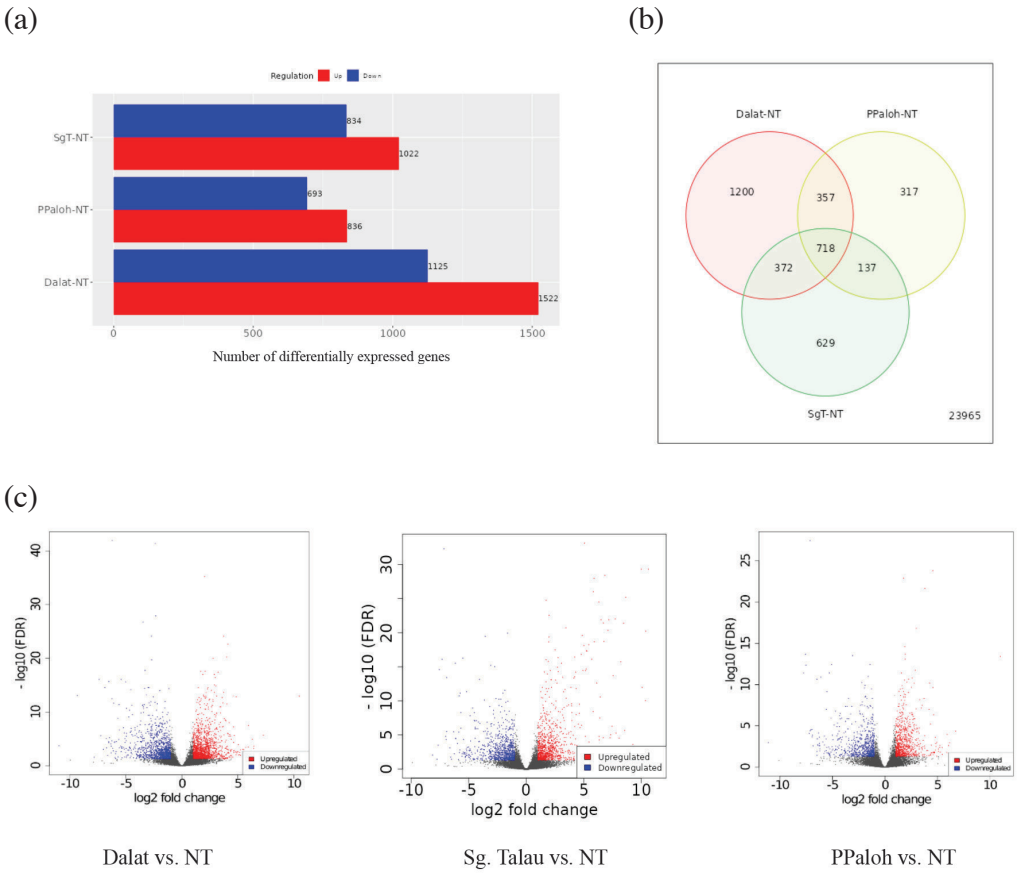

Figure 3: DEG expression of sago palms from different locations against NT. (a) Number of DEGs between samples, (b) Venn diagram between samples, and (c) volcano plot of DEGs between different groups

As for the molecular function category, inorganic cation transmembrane transporter activity (GO: 0022890), ubiquitin protein ligase activity (GO: 0004842), ubiquitin-like protein ligase activity (GO: 0061659), and monoatomic cation transmembrane transporter activity (GO: 0008324) were significantly enriched GO terms in the upregulated DEGs. O-methyltransferase activity (GO: 0008171), oxidoreductase activity, acting on peroxide as acceptor (GO: 0016684), and peroxidase activity (GO: 0004601) were significantly enriched GO terms in the downregulated DEGs of the comparative datasets.

The commonly expressed DEGs were assigned to the KEGG pathways enrichment analysis. It was revealed the pathways essential for plant growth, development, and adaptation such as the kinase modules transducers; Mitogen-Activated Protein Kinase (MAPK) pathways and ribosome were enriched in upregulated DEGs. The orchestration of MAPK cascades amplifies extracellular stimuli response and relays the signals downstream influencing gene expression to enhance plant adaptation and cell survival as well as cell proliferation and differentiation (Xie *et al.*, 2023). The ribosome is responsible in protein translation and synthesis. Any defects or reduction in ribosome biogenesis level can result in cell differentiation and developmental

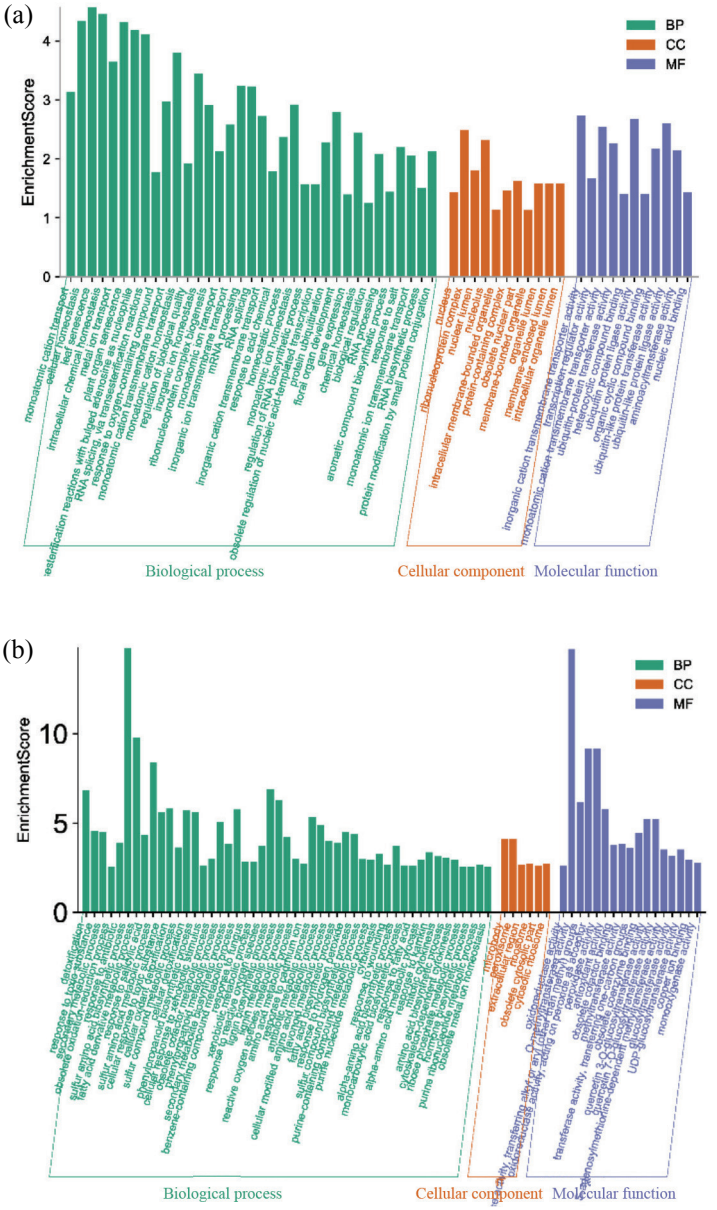

Figure 4: GO classification analyses in upregulated (a) and downregulated (b) commonly expressed DEGs between three comparative datasets

defects (Breznak *et al.*, 2023). On the other hand, the pathways associated with the accumulation of secondary metabolites such as the metabolism for other secondary amino acids, biosynthesis of other secondary metabolites, and metabolism were enriched in downregulated DEGs. The plant secondary metabolites such as terpenes, polysaccharides, flavonoids, and phenolic compounds were documented to play a crucial role in plant defence mechanism against environmental stresses but not in plant growth (Khare *et al.*, 2020). Figure 5 shows the

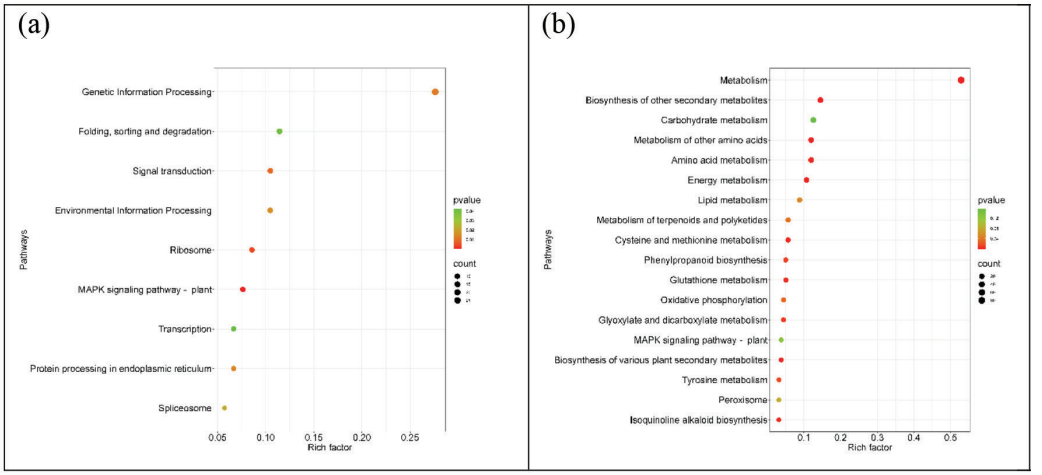

Figure 5: KEGG pathway enrichment analysis of the DEGs in the three comparative sets. (a) KEGG scatter plot enrichment of upregulated DEGs and (b) KEGG scatter plot enrichment of downregulated DEGs

scatter plot enrichment of both upregulated and downregulated DEGs.

Transcription Factors Related to Commonly Expressed DEGs

A homology search of 718 DEGs with Arabidopsis genes identified 17 TF families, with most TFs belonging to the WRKY and ERF families. Other expressed putative TFs were NAC, TCP, MYB, MYB-related, and bHLH.

Discussions

Plants are sessile. To ensure their survival and normal development, plants must have the necessary systems to counteract countless effects from biotic and abiotic stressors. Hence, it is not surprising that most agricultural breeding programmes are geared toward producing stress-tolerant plant varieties (Cohen & Leach, 2019). The molecular mechanisms underlying the occurrences of trunking and non-trunking in sago palms are still poorly understood. In this study, a collection of trunking sago palms from different locations grown on different types of soils including peat and mineral soils were sequenced using RNA-seq platform. The transcriptome sequencing obtained a sequence data of 85.60 Gb, significantly promoting understanding on the functional transcriptome of *M. sagu*. By comparing leaf transcriptome from trunking and non-trunking sago palms, this study established the transcriptome DEGs contributing to the sago palm development. The KEGG pathways of 718 common DEGs revealed the MAPK signalling pathway, ribosome, and plant signal transduction pathway, as well as phenylpropanoid biosynthesis pathways were found commonly enriched in trunking sago palms grown in peat and mineral soils.

The MAPK cascades are important signalling modules that transmit signals from receptors to convert into cellular responses. The MAPK pathways in plants have always been linked to a variety of abiotic and biotic stress responses (Jagodzik *et al.*, 2018). The phosphorylation of the MAPK tiers can influence plant growth and development through the varied roles of the downstream cascades (Chang & Lv, 2023). One of the transcripts related to the MAPK signalling pathway in this study was the mitogen-activated protein kinase kinase (MAPKK kinase 17/18) (AT1G05100). This MAPK ERK KINASE KINASE (MEKK) subfamily was reported to enhance drought resistant in plants. Studies on Arabidopsis reported that abscisic acid (ABA) signal induction upon osmotic stress induced

Arabidopsis *MAPKKK18*, which brings about drought resistant to *A. thaliana* via downstream MKK3. Loss-of-function among Arabidopsis mapkkk18 mutants were found severely sensitive to drought. Hence, this confirms the necessity of *MAPKKK18* in accelerating leaf stomatal closure to reduce water transpirational rate during osmotic stress (Li *et al.*, 2017). Moreover, the *MAPKKK18* also participate in leaf senescence. Mutants *MAPKKK18* displayed delayed senescence compared with the wild type Arabidopsis while *MAPKKK18* overexpression lines displayed accelerated leaf senescence (G. Zhao *et al.*, 2023). Leaf senescence is another key mechanism for plant survival during severe or prolonged drought stress. Leaf senescence prevents xylem cavitation which can reduce xylem hydraulic conductance. Reduced water transport upward from soil to leaves result in cambium and apical meristem dehydration, canopy dieback, and ultimately tree death (Nadal-Sala *et al.*, 2021).

Another MAPK signalling pathway transcript expressed was the MAPK kinase 9 (MKK9) (AT1G73500). The MKK9 is an upstream activator of the Mitogen-activated Protein Kinase (MPKs) MPK3 and MPK6. The MKK9 was found activated in plants grown under limited nitrogen (N) and phosphorus (Pi) conditions. Studies conducted on Arabidopsis grown under N- and Pi limiting reported that *MKK9* expression modulated the downstream cascades of MPK3/MPK6 with transcription factor WRKY 75 to activate responsible genes to increase N and Pi acquisition and accumulation in plants. The activation of MKK9 negatively regulates anthocyanin biosynthesis in the stressed plants (Lei *et al*., 2014; Luo *et al*., 2017). This condition was also reported in the study of apple in response to different N level (Sun *et al.*, 2022). The upregulation of *MAPKKK18* and *MAPKK9* suggests that the studied plants were experiencing drought stress and other soil-related constraints such as nutrient deficiency.

Ribosome biogenesis is responsible for cell proliferation. The fine tune translation of Ribosomal Proteins (RPs) synthesising varying protein levels facilitate protein synthesis for cell growth and assist plants in stress situation response (Dong *et al.*, 2015; Lan *et al.*, 2022). In this study, the commonly expressed DEGs transcribed large ribosomal subunits (RPLs) in the ribosome pathway. Studies reported that the RPL gene family is crucial in stress alleviation.

Studies have pointed out that any mutations in the ribosomal proteins result in reduced global protein translation levels and caused detrimental effects on plant growth and development such as retarded root growth, vascular pattern defects, late flowering, and reduced plant size (Byrne, 2009; Fakih *et al*., 2023). The upregulation of RPL27A genes in all datasets suggests better stress response among trunking sago palms compared with the NT; for instance, the study on rpl27a mutant in Arabidopsis was observed to cause a "pointed", and serrated initial leaf and restrict plant growth (Moin *et al.*, 2016). Hence, it be can postulated that the upregulation of RPL27A in trunking sago palms helps in the palms stress response through the biosynthesis of stress-alleviation related proteins.

The plant hormone signal transduction pathway is another important regulator of plant growth and development. The DEGs transcribed in this pathway play a role in the tryptophan metabolism pathway, carotenoid biosynthetic pathway, and α-linolenic acid metabolism pathway. The auxin response factor (ARF) (AT1G19850), jasmonate ZIM domain-containing protein, SAUR family protein (AT1G16510), and abscisic acid receptor PYR/PYL family (AT1G01360) as well as those genes transcribed in MAPK signalling pathways such as *MAPKKK18* and *MKK9* were involved in this pathway. The ARF and SAUR are known as Auxin Early Response Genes (AERGs). Studies reported that ARFs and JAZ were found responsible in regulating drought and osmotic stress in plants; for example, *Juglan sigillata JsARF28* (Wang *et al.*, 2024), tomato *SlARF4* (Bouzroud *et al.*, 2020), rice *OsARF11* and *OsARF15* (Song *et al.*, 2023), and poplar *JASMONATE ZIM-domain protein 2* (*PeJAZ2*) (Rao *et al.*, 2023) were found upregulated in drought and osmotic stress environments. The

expression of the ARFs and JAZ resulted in reduced stomatal conductance in leaves and increase relative water content (Kou *et al.*, 2022). The *SAUR* gene family is involved in cell expansion and also drought stress. Studies documented *AtSAUR19/24* involved in leaf development (Spartz *et al.*, 2012) whereas *AtSAUR32* enhanced drought tolerance (He *et al.*, 2021). The remaining genes were involved in plant response to biotic stressors like fungal infection. For example, jasmonic acid coordinates growth and defence response upon fungal infection in poplar and *Zea mays* (Luo *et al.*, 2019). Based on these pathways, it can be postulated the trunking sago palms have better response toward salinity stress.

Most of the KEGG pathways presented by the downregulated DEGs belonged to the main metabolism class. Among the enriched KEGG pathways were metabolism, secondary metabolite biosynthesis, amino acid metabolism, energy metabolism, cysteine and methionine metabolism, and phenylpropanoid biosynthesis. However, only the phenylpropanoid biosynthesis pathway is discussed further due to the extensive content of secondary metabolite metabolism, amino acid metabolism and energy metabolism biosynthesis. The genes transcribed enzymes cinnamyl-alcohol dehydrogenase (CAD), caffeoyl-CoA O-methyltransferase, 4-coumarate—CoA ligase (4CL), and 5-O-(4-coumaroyl)-D-quinate 3'-monooxygenase were found involved in the phenylpropanoid biosynthesis pathway. These enzymes are involved in the monolignol biosynthesis (M00039). Lignin is a crucial polymer that allows plants to develop taller by giving mechanical supports to xylem for it to endure pressure caused by water transfer from roots to shoots. Li and Chapple (2010) reported that lignin-deficient plants frequently exhibit reduced growth and dwarfism due to xylem failure to transport water. Several studies have demonstrated that a decrease in lignin is compensated for by an increase in cellulose, as denoted in poplars with downregulation of 4-coumarate: CoA ligase (4CL) and caffeic acid O-methyltransferase (COMT) (Van Acker *et al.*, 2013). Despite the reduction of lignin concentration in mutants *Arabidopsis* there were no visible morphological abnormalities, nevertheless the plants retained a normal phenotype (Hu *et al*., 1999; Van Acker *et al*., 2013). Furthermore, lignin reductions are usually compensated for increases in cellulose content and substantially increased growth in plants (Hu *et al.*, 1999). The downregulation of monolignol synthesis in the sago palm may be associated with the palm's *Pelawei manit* growth stage, where the palm bulks its starch content in its trunk.

The Transcription Factors (TFs) families identified were associated with plant response to abiotic and biotic stresses. The ability to properly address cellular networks guarantees their survival (Takahashi & Shinozaki, 2019). Transcription factors function pivotally in signal transduction to activate or suppress gene expression, regulating the interaction between different signalling pathways (Weidemüller *et al.*, 2021). In this study, WRKY and ERF TF families were overrepresented in the shared 718 DEGs of the three comparative sets.

The WRKY TFs are one of the largest families of transcription regulators found exclusively in plants. Research has shown that WRKY interacts with other transcription factors either by acting as activators or repressors to form signalling webs that contribute to various cytoplasmic and nuclear processes to regulate defence-related genes hence establishing their significant importance in plant stress response (Wani *et al.*, 2021). Research bodies documented that WRKY TFs are essential in plant adaptation to abiotic and biotic stresses. In fact, MAP kinase signalling cascade is one of the major signalling pathways which involves WRKY TFs to address abiotic and biotic stresses. An array of studies on salt/salinity and drought response reported that WRKY TFs improved salt and drought response by providing better Reactive Oxygen Species (ROS) scavenging performance (S. Wang *et al*., 2015; Agarwal *et al*., 2016). Transformation of wheat *WRKY* genes i.e., *WRKY1*, *WRKY2*, and *WRKY33* into Arabidopsis (G.-H. He *et al*., 2016; Gao *et al*., 2018) and *Malus baccata WRKY2* and *WRKY3* into tobacco

(Han *et al.*, 2018) enhance drought and salt tolerance by inducing stomatal closure and reducing transpiration rate. Further, transgenic *Arabidopsis* overexpressing *WRKY46* from *Fagopyrum tataricum* (*Ftwrky46*) (Lv *et al.*, 2020) and transformation of horse gram (*Macrotyloma uniflorum*) WRKY3 gene into groundnut (Kiranmai *et al.*, 2018) improved drought tolerance of Arabidopsis and groundnut by enhancing osmoprotectants i.e., proline and soluble sugar (Blum, 2017) and decrease accumulation of malondialdehyde (MDA) content (Khan *et al.*, 2019). Unlike most salt response studies, WRKY TFs were found upregulated to enhance salt sensitivity of the plant, this study observed downregulation of WRKY genes. Likewise, salt response study on *Brachypodium distachyon* found most of the *WRKYs* were downregulated when salt stress was inflicted (Wen *et al.*, 2014). Meanwhile, in soybean, Yu *et al.* (2016) reported transient response of some *WRKY* genes on salt stress. For example, *WRKY44*, *-51*, *-54*, *-78*, *-81*, *-102*, and *-107* transcripts peaked after six hours of high-concentration salt exposure and decreased shortly after. The study also reported that most *WRKYs* are upregulated in root and are downregulated in aerial parts of the plants, hence, reflecting major degree of functional divergence of the *WRKY* gene family. Xiang *et al.* (2021) explained that the *WRKY* genes found downregulated upon salt stress are the group III of WRKY TF i.e., *AtWRKY70* and *AtWRKY54* are negative regulators in osmotic stress tolerance (Li *et al.*, 2013) and was reported in salt-treated *Populus* (*PsnWRKY70*) (Zhao *et al.*, 2015) and tomato (*SlWRKY70*) (Abbasi *et al.*, 2020).

Ethylene Response Factors (ERFs) are one of the largest subfamilies of Apetala2 (AP2)/ERF transcription factor responsible for modulating multiple responses against abiotic and biotic stresses (Wang *et al*., 2014; Thirugnanasambantham *et al*., 2015). ERF family act as the central part of the plant signal transduction network through the induction of phytohormones such as ethylene, jasmine, ABA salicylic, and environmental cues, namely salinity stress, drought stress, temperature stress, and submergence tolerance (Liu *et al.*, 2012). Interestingly, ERF TFs are a double-edged sword. Thirugnanasambantham *et al.* (2015) explained that despite most ERFs acting as activators of stress-responsive genes, certain ERFs could act as repressors; for example, pathogen-inducible plant defensin (PDF1.2) is known to be activated by the ERF proteins (ERF1 and ERF2). The ERF3 and ERF4 repress gene expression and plant defence systems (Maruyama *et al.*, 2013). Similarly, overexpression of VpERF2 and VpERF3 in transgenic tobacco enhanced resistance to a bacterial pathogen (*Ralstonia solanacearum*) and fungal pathogen (*Phytophtora parasitica*). However, overexpression of VpERF1 increased the susceptibility of both pathogens (Zhu *et al.*, 2013). This event is also reported in Chinese cabbage (Lai *et al.*, 2013) and tomato (Cao *et al.*, 2006). Besides protecting against biotic stresses, ERFs play critical protective roles against abiotic stresses. In rice, overexpression of ERF DREB transcription factors, SALT-RESPONSIVE ERF1 (SERF1) enhanced salt tolerance (Hong *et al.*, 2022). Similarly, overexpression of ERFs was also reported to enhance salt tolerance in Arabidopsis, wheat, and *Glycine max* whereby the AP2/EREBP family TFs were found to increase wax content, reduce stomatal density, and alter cuticle properties mutant *Arabidopsis shine* (*shn*) (Aharoni *et al.*, 2004). Flooding is another severe abiotic stress that can negatively impact plant growth and development. It causes soil hypoxia, a condition that hinders gas exchange between plants and the environment. This condition leads to the accumulation of ROS and triggering oxidative stress, hence, resulting in premature death among the affected plants. Ethylene, a type of phytohormone plays an array of roles in plants including plant response, adaptation improvement to plant survival. Flooding and submergence accelerate ethylene accumulation and regulates the ERFs group VII (ERFVIII) transcription factor family; for example, in rice, the ethylene-related genes *SUBMERGENCE 1* (*SUB1*) encoding *SUB1A*, *SUB1*, and *SUB1C* prolong the survival of

submerged rice up to 14 days as compared to rice accessions lacking *SUB1A* gene (Xu *et al*., 2006; Alpuerto *et al*., 2022). Moreover, the rapid accumulation of ethylene during plant submergence stimulates the accumulation of two group of ERFVII genes: *SNORKEL1* (SK1) and *SNORKEL2* (SK2) causing elongation of the rice stem internodes (Hattori *et al*., 2009; Hattori *et al*., 2011).

Limitations and Recommendations for Future Studies

The present study reveals valuable insight to the MAPKK pathway related to drought and osmotic stress in sago palms. However, several limitations warrant consideration for future research. The study lacks in root transcriptome datasets from both trunking and NT sago palms. The absence of root transcriptome data restricts comprehensive understanding of drought and osmotic stress responses from these palms. Root transcriptome serve as the plant first line of defence against soil stresses, making this analysis crucial to brings insight into soil-specific responses and interplay between roots and soil rhizosphere environment. Future research can also study soil taxonomy. Conducting soil taxonomy investigation with amplicon sequencing is beneficial to identify putative microorganisms that provide resilience to the tree and growth enhancement. In summary, these suggested investigations will provide a holistic understanding of transcriptomes in both roots and leaves to decipher putative responsible genes in governing sago palm growth and development. Addressing these limitations will pave the way for a more robust and impactful research in this field.

Conclusions

The comparative datasets of trunking sago palms from different locations against NT datasets yield 718 commonly expressed genes. Enrichment analyses with KEGG pathways showed that these DEGs are responsible towards the resilience of trunking sago palms against several environmental stresses such as drought and nutrient deficiency stress, as denoted by the regulation of MAPK pathways and ribosome production.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Appendix

Table S1: Statistics of the quality and outpt of the RNA-Seq libraries

Samples	Raw Reads (M)	Clean Reads (M)	Clean Bases (Gb)	Q20 (%)	Q30 (%)	GC Content (%)	Mean Length (Bp)
Dalat_P1a	41.64	41.36	6.19	97.85	94.80	48.98	149.00
Dalat_P1b	45.49	45.19	6.76	97.55	92.97	48.97	149.00
Dalat_P2	56.31	55.92	8.36	97.77	93.55	49.40	149.00
Dalat_P3	101.45	100.74	15.10	97.53	93.04	48.91	149.00
PayaPaloh_P1	42.37	42.12	6.31	97.93	93.92	49.20	149.00
PayaPaloh_P2	41.74	41.48	6.21	97.96	94.01	48.39	149.00
PayaPaloh_P3	41.82	41.49	6.20	97.70	93.43	49.28	149.00
Sg. Talau_P1	43.08	42.78	6.40	97.94	94.03	49.13	149.00
Sg. Talau_P2a	42.43	42.21	6.31	98.31	94.84	49.52	149.00
Sg. Talau_P2b	41.26	41.01	6.13	97.91	93.91	50.10	149.00
Sg. Talau_P3	78.42	77.71	11.65	96.93	91.74	49.49	149.00
			85.61				

Table S2: Gene Ontology (GO) enrichment analysis of 718 DEGs

The Upregulated DEGs						
Category	**Description**	**GO_ID**		**Enrichment Score**	**Hits Genes Counts in Selected Set**	**Corrected *p*-value (BH Method)**
Biological process	Monoatomic cation transport	GO:0006812	0.0021333	3.1381484	9	0.2402678
Biological process	Cellular homeostasis	GO:0019725	0.00256	4.3376724	6	0.2096875
Biological process	Leaf senescence	GO:0010150	0.0047373	4.566899	5	0.284555
Biological process	Intracellular chemical homeostasis	GO:0055082	0.005242	4.4581633	5	0.295191
Biological process	Metal ion transport	GO:0030001	0.0059617	3.6475881	6	0.2984151
Biological process	Plant organ senescence	GO:0090693	0.0060112	4.3143515	5	0.2850571
Biological process	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	GO:0000377	0.0068569	4.1795281	5	0.3089028
Biological process	RNA splicing, via transesterification reactions	GO:0000375	0.007376	4.106203	5	0.3020789
Biological process	Response to oxygen-containing compound	GO:1901700	0.0074728	1.7752566	19	0.292738
Biological process	Monoatomic cation transmembrane transport	GO:0098655	0.0089962	2.9721088	7	0.3117545
Biological process	Monoatomic cation homeostasis	GO:0055080	0.0100547	3.8057491	5	0.3123885
Biological process	Regulation of biological quality	GO:0065008	0.0122231	1.9176298	14	0.3441561
Biological process	Inorganic ion homeostasis	GO:0098771	0.0150076	3.4419643	5	0.3654561
Biological process	Ribonucleoprotein complex biogenesis	GO:0022613	0.01689	2.9105107	6	0.3308243
Biological process	Monoatomic ion transport	GO:0006811	0.0179013	2.1277597	10	0.3360222
Biological process	Inorganic ion transmembrane transport	GO:0098660	0.0184111	2.5801181	7	0.325262
Biological process	Mrna processing	GO:0006397	0.0190079	3.2394958	5	0.3231345

Biological process	RNA splicing	GO:0008380	0.0192631	3.2283251	5	0.3214083
Biological process	Inorganic cation transmembrane transport	GO:0098662	0.0224883	2.7268377	6	0.311722
Biological process	Response to acid chemical	GO:0001101	0.0267578	1.7871932	13	0.3090865
Biological process	Homeostatic process	GO:0042592	0.0277349	2.3701627	7	0.3163185
Biological process	Monoatomic ion homeostasis	GO:0050801	0.0283271	2.9165554	5	0.3150955
Biological process	Regulation of RNA biosynthetic process	GO:2001141	0.0300459	1.5720016	18	0.3261604
Biological process	Obsolete regulation of nucleic acid-templated transcription	GO:1903506	0.0300459	1.5720016	18	0.3261604
Biological process	Protein ubiquitination	GO:0016567	0.0333007	2.2794783	7	0.333377
Biological process	Floral organ development	GO:0048437	0.0335479	2.786352	5	0.3321611
Biological process	Gene expression	GO:0010467	0.035059	1.3929368	26	0.3396572
Biological process	Chemical homeostasis	GO:0048878	0.0358886	2.4422981	6	0.3439959
Biological process	Biological regulation	GO:0065007	0.0370813	1.2474541	40	0.3341027
Biological process	RNA processing	GO:0006396	0.0373964	2.0804762	8	0.3336051
Biological process	Aromatic compound biosynthetic process	GO:0019438	0.038894	1.4423469	22	0.3402278
Biological process	Response to salt	GO:1902074	0.0395752	2.1954774	7	0.3363895
Biological process	Monoatomic ion transmembrane transport	GO:0034220	0.0403379	2.0491694	8	0.339668
Biological process	RNA biosynthetic process	GO:0032774	0.0451123	1.5006106	18	0.3415647
Biological process	Protein modification by small protein conjugation	GO:0032446	0.0462522	2.1208738	7	0.3472767
Cellular component	Nucleus	GO:0005634	0.0046336	1.4337084	38	0.1644932
Cellular component	Ribonucleoprotein complex	GO:1990904	0.0064768	2.489466	10	0.1724458
Cellular component	Nuclear lumen	GO:0031981	0.0167794	1.800277	15	0.2102361
Cellular component	Nucleolus	GO:0005730	0.0217424	2.315077	8	0.2572851

Cellular component	Intracellular membrane-bounded organelle	GO:0043231	0.0300561	1.1358484	63	0.266748
Cellular component	Protein-containing complex	GO:0032991	0.0304806	1.4655757	23	0.2497062
Cellular component	Obsolete nuclear part	GO:0044428	0.0316774	1.6288221	16	0.2409744
Cellular component	Membrane-bounded organelle	GO:0043227	0.0356148	1.1296559	63	0.2528647
Cellular component	Organelle lumen	GO:0043233	0.0477609	1.5767638	15	0.2992079
Cellular component	Membrane-enclosed lumen	GO:0031974	0.0477609	1.5767638	15	0.2992079
Cellular component	Intracellular organelle lumen	GO:0070013	0.0477609	1.5767638	15	0.2992079
Molecular function	Inorganic cation transmembrane transporter activity	GO:0022890	0.0220716	2.7355392	6	0.3232834
Molecular function	Transcription regulator activity	GO:0140110	0.025389	1.6642064	16	0.274864
Molecular function	Monoatomic cation transmembrane transporter activity	GO:0008324	0.030097	2.5446876	6	0.3122563
Molecular function	Ubiquitin-protein transferase activity	GO:0004842	0.0346841	2.2572229	7	0.3084412
Molecular function	Heterocyclic compound binding	GO:1901363	0.0383418	1.4083577	24	0.298347
Molecular function	Ubiquitin protein ligase activity	GO:0061630	0.0388948	2.6731654	5	0.2848469
Molecular function	Organic cyclic compound binding	GO:0097159	0.0410826	1.3993522	24	0.2922734
Molecular function	Ubiquitin-like protein transferase activity	GO:0019787	0.0417383	2.1677858	7	0.2886901
Molecular function	Ubiquitin-like protein ligase activity	GO:0061659	0.0431618	2.5970309	5	0.2621289
Molecular function	Aminoacyltransferase activity	GO:0016755	0.0442849	2.1395279	7	0.2625462
Molecular function	Nucleic acid binding	GO:0003676	0.0478171	1.4272379	21	0.2645877

The Downregulated DEGs						
Category	**Description**	**GO-ID**	**Enrichment Score**		**Hits Genes Counts in Selected Set**	**Corrected *p*-value (BH Method)**
Biological process	Detoxification	GO:0098754	5.94E-07	6.8531705	11	8.06E-04
Biological process	Response to toxic substance	GO:0009636	1.05E-06	4.5424195	15	7.13E-04
Biological process	Secondary metabolic process	GO:0019748	1.18E-06	4.5006181	15	5.33E-04
Biological process	Obsolete oxidation-reduction process	GO:0055114	5.86E-06	2.527237	27	0.0015881
Biological process	Response to antibiotic	GO:0046677	6.59E-06	3.9125373	15	0.0014895
Biological process	Sulfur amino acid biosynthetic process	GO:0000097	1.95E-05	14.820217	5	0.0029444
Biological process	Fatty acid derivative metabolic process	GO:1901568	3.25E-05	9.7813433	6	0.0040048
Biological process	Response to salicylic acid	GO:0009751	4.72E-05	4.338499	11	0.0053366
Biological process	Cellular oxidant detoxification	GO:0098869	2.05E-04	5.8024918	7	0.0139001
Biological process	Sulfur compound metabolic process	GO:0006790	2.27E-04	3.6349587	11	0.0146351
Biological process	Cellular detoxification	GO:1990748	2.27E-04	5.7057836	7	0.0140193
Biological process	Phenylpropanoid biosynthetic process	GO:0009699	2.52E-04	5.6122461	7	0.0148469
Biological process	Cellular response to xenobiotic stimulus	GO:0071466	3.87E-04	2.6214655	16	0.0187407
Biological process	Phenylpropanoid metabolic process	GO:0009698	4.88E-04	5.0345149	7	0.0206615
Biological process	Secondary metabolite biosynthetic process	GO:0044550	5.89E-04	3.8274822	9	0.0242004
Biological process	Benzene-containing compound metabolic process	GO:0042537	6.16E-04	5.7537313	6	0.0245584
Biological process	Response to fungus	GO:0009620	6.73E-04	2.8383362	13	0.0260759
Biological process	Xenobiotic metabolic process	GO:0006805	7.46E-04	2.807008	13	0.0281001

Biological process	Response to reactive oxygen species	GO:0000302	7.52E-04	3.6988273	9	0.027567
Biological process	Lignin biosynthetic process	GO:0009809	7.86E-04	6.8882699	5	0.0280473
Biological process	Reactive oxygen species metabolic process	GO:0072593	0.001417	4.2005769	7	0.0400309
Biological process	Amino acid metabolic process	GO:0006520	0.0018901	3.0004121	10	0.0466004
Biological process	Response to cadmium ion	GO:0046686	0.0024662	2.7170398	11	0.0539375
Biological process	Antibiotic metabolic process	GO:0016999	0.0025033	5.3159474	5	0.053881
Biological process	Cellular modified amino acid metabolic process	GO:0006575	0.0035921	4.8906716	5	0.0632591
Biological process	Flavonoid metabolic process	GO:0009812	0.0039817	3.992385	6	0.0666569
Biological process	Fatty acid biosynthetic process	GO:0006633	0.0043942	3.9125373	6	0.0700998
Biological process	Response to external biotic stimulus	GO:0043207	0.004465	1.779833	23	0.0695917
Biological process	Response to biotic stimulus	GO:0009607	0.0046407	1.7742184	23	0.0707055
Biological process	Response to hydrogen peroxide	GO:0042542	0.0051832	4.4868547	5	0.0763964
Biological process	Sulfur compound biosynthetic process	GO:0044272	0.0055952	4.4060105	5	0.0807133
Biological process	Purine-containing compound metabolic process	GO:0072521	0.0056045	2.9753135	8	0.0799971
Biological process	Purine nucleotide metabolic process	GO:0006163	0.0095743	2.9640434	7	0.1180256
Biological process	Cytokinesis	GO:0000910	0.0101635	3.2786626	6	0.113898
Biological process	Response to wounding	GO:0009611	0.0102437	2.6798201	8	0.112931
Biological process	Alpha-amino acid biosynthetic process	GO:1901607	0.011404	3.7050543	5	0.1171507
Biological process	Response to endogenous stimulus	GO:0009719	0.0114442	1.5172715	30	0.1166792
Biological process	Monocarboxylic acid biosynthetic process	GO:0072330	0.0116998	2.6170818	8	0.1183949
Biological process	Response to fatty acid	GO:0070542	0.0123685	2.5910843	8	0.1233216

Biological process	Alpha-amino acid metabolic process	GO:1901605	0.0160653	2.9640434	6	0.1492095
Biological process	Mitotic cytokinesis	GO:0000281	0.0219565	3.1350459	5	0.1782818
Biological process	Amino acid biosynthetic process	GO:0008652	0.0241767	3.0566698	5	0.1895004
Biological process	Cytoskeleton-dependent cytokinesis	GO:0061640	0.0271492	2.9640434	5	0.2068222
Biological process	Ribose phosphate metabolic process	GO:0019693	0.030736	2.5516548	6	0.2104951
Biological process	Hormone biosynthetic process	GO:0042446	0.0318714	2.5296577	6	0.2171739
Biological process	Purine ribonucleotide metabolic process	GO:0009150	0.0405205	2.6579737	5	0.2298989
Biological process	Obsolete metal ion homeostasis	GO:0055065	0.0462999	2.5605611	5	0.2562558
Cellular component	Microbody	GO:0042579	3.52e-04	4.1046316	9	0.0371719
Cellular component	Peroxisome	GO:0005777	3.52e-04	4.1046316	9	0.0371719
Cellular component	Extracellular region	GO:0005576	0.0018569	2.6605714	12	0.0783605
Cellular component	Ribosome	GO:0005840	0.0095829	2.7096808	8	0.1347994
Cellular component	Obsolete cytosolic part	GO:0044445	0.0116817	2.6159497	8	0.1449908
Cellular component	Cytosolic ribosome	GO:0022626	0.0148856	2.716	7	0.1744921
Molecular function	Oxidoreductase activity	GO:0016491	3.99e-06	2.6253574	26	0.0015615
Molecular function	O-methyltransferase activity	GO:0008171	1.95e-05	14.75542	5	0.0012676
Molecular function	Transferase activity, transferring alkyl or aryl (other than ,ethyl) groups	GO:0016765	1.39E-04	6.1575504	7	0.0067796
Molecular function	Oxidoreductase activity, acting on peroxide as acceptor	GO:0016684	2.06e-04	9.1483607	5	0.0089287
Molecular function	Peroxidase activity	GO:0004601	2.06e-04	9.1483607	5	0.0089287
Molecular function	Antioxidant activity	GO:0016209	2.08e-04	5.7692365	7	0.0074022
Molecular function	Obsolete cofactor binding	GO:0048037	3.14e-04	3.7803143	10	0.0094386
Molecular function	Methyltransferase activity	GO:0008168	0.0011998	3.8118169	8	0.0234561
Molecular function	Transferase activity, transferring one-carbon groups	GO:0016741	0.0017102	3.6052653	8	0.0318424

Molecular function	Obsolete coenzyme binding	GO:0050662	0.0022646	4.462615	6	0.0354183
Molecular function	Quercetin 3-o- glucosyltransferase activity	GO:0080043	0.0027334	5.1979322	5	0.0368539
Molecular function	Quercetin 7-o- glucosyltransferase activity	GO:0080044	0.0027334	5.1979322	5	0.0368539
Molecular Function	S-Adenosylmethionine- dependent methyltransferase activity	GO:0008757	0.0072417	3.5186003	6	0.0745133
Molecular function	Glucosyltransferase activity	GO:0046527	0.0120337	3.1546071	6	0.1022866
Molecular function	Udp-glucosyltransferase activity	GO:0035251	0.0139224	3.5186003	5	0.1134097
Molecular function	Copper ion binding	GO:0005507	0.0281274	2.9321669	5	0.1666338
Molecular function	Monooxygenase activity	GO:0004497	0.034621	2.7722305	5	0.1781161

Table S3: KEGG pathway enrichment analysis of 718 DEGs

The Upregulated DEGs						
KEGG Pathway	**Main Class**	**Gene Hits in Selected Set**	**All Genes in Selected Set**	***p*-value**	**Corrected *p*-value (BH Method)**	**Enrichment Factor**
04016 MAPK signaling pathway - plant	A09130 Environmental Information Processing	8	105	2.00E-04	0.023377	5.00387
03010 Ribosome	A09120 Genetic Information Processing	9	105	0.00354	0.08284	2.942312
B 09132 Signal transduction	A09130 Environmental Information Processing	11	105	0.008511	0.124474	2.289955
A09120 Genetic information processing	A09120 Genetic Information Processing	29	105	0.01155	0.135134	1.51389

04141 Protein processing in endoplasmic reticulum	A09120 Genetic Information Processing	7	105	0.012993	0.138196	2.787403
A09130 Environmental information processing	A09130 Environmental Information Processing	11	105	0.015446	0.150601	2.101521
03040 Spliceosome	A09120 Genetic Information Processing	6	105	0.021634	0.180795	2.767292
B 09123 Folding, sorting and degradation	A09120 Genetic Information Processing	12	105	0.04106	0.300255	1.748693
B 09121 Transcription	A09120 Genetic Information Processing	7	105	0.042535	0.276478	2.174301

The Downregulated DEGs

KEGG Pathway	Main Class	Gene Hits in Selected Set	All Genes in Selected Set	*p*-value	Corrected *p*-value (BH Method)	Enrichment Factor
A09100 Metabolism	A09100 Metabolism	84	159	2.73E-13	3.91E-11	2.046476
B 09110 Biosynthesis of other secondary metabolites	A09100 Metabolism	23	159	7.34E-11	5.25E-09	5.240705
B 09106 Metabolism of other amino acids	A09100 Metabolism	19	159	1.13E-09	5.37E-08	5.635158
B 09102 Energy metabolism	A09100 Metabolism	17	159	2.14E-05	7.65E-04	3.22391
00999 Biosynthesis of various plant secondary metabolites	A09100 Metabolism	6	159	4.61E-05	0.001319	9.199232

00950 Isoquinoline alkaloid biosynthesis	A09100 Metabolism	5	159	9.48E-05	0.002259	10.76894
B 09105 Amino acid metabolism	A09100 Metabolism	19	159	1.18E-04	0.002112	2.616017
00270 Cysteine and methionine metabolism	A09100 Metabolism	9	159	1.42E-04	0.002028	4.652183
00480 Glutathione metabolism	A09100 Metabolism	8	159	1.88E-04	0.002446	5.06065
00630 Glyoxylate and dicarboxylate metabolism	A09100 Metabolism	7	159	5.46E-04	0.006508	4.946983
00940 Phenylpropanoid biosynthesis	A09100 Metabolism	8	159	9.42E-04	0.009623	3.976225
00350 Tyrosine metabolism	A09100 Metabolism	5	159	0.001271	0.012119	6.19583
00190 Oxidative phosphorylation	A09100 Metabolism	7	159	0.003876	0.027716	3.517855
B 09109 Metabolism of terpenoids and polyketides	A09100 Metabolism	9	159	0.005199	0.035402	2.788124
B 09103 Lipid metabolism	A09100 Metabolism	14	159	0.008298	0.05159	2.059232
04146 Peroxisome	A09140 Cellular processes	5	159	0.017539	0.080905	3.325703
04016 MAPK signaling pathway - plant	A09130 Environmental information processing	6	159	0.03487	0.131221	2.478332
B 09101 Carbohydrate metabolism	A09100 Metabolism	20	159	0.042927	0.157398	1.503892