

Carbohydrate deprivation upsurges the expression of genes responsible for programmed cell death in inflorescence tissues of oil palm (*Elaeis guineensis* Jacq.)

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Abstract: The aim of this present study was to analyze global gene expression from a developing inflorescence of oil palm under complete defoliation stress. We used the Illumina platform to sequence six cDNA libraries of defoliated and nondefoliated tissues. The frequency of differentially expressed genes (DEGs) decreased with increasing fold change at $P \leq 0.05$. Gene ontology analysis revealed that about 15% of all DEGs were responsible for stress response while 13.9% of genes were responsible for cell organization and developmental processes. DAVID functional analysis clustering of DEGs identified a higher enrichment score (ES) for cellular response to stress (ES = 3.2%), response to abiotic stress (ES = 3.12%), cell cycle (ES = 2.25%), and response to carbohydrate stimulus (ES = 1.34%), signifying the functional implications of these processes in carbohydrate depletion and flower development. The top ten DEGs were principally responsible for stress response and cell death. There was a high expression of the *SEC14*, *TIFY8*, *NUDT7*, and *DUF538* genes that are related to cell death. It is necessary to further investigate the role of these genes in premature inflorescence abortion in oil palm during stress.

Key words: Complete defoliation, stress response, differentially expressed genes, RNA-seq, carbohydrate, oil palm

1. Introduction

Oil palm is an important economic tropical and semitropical crop covering more than 15×10^6 ha planted area and representing 5% of total world vegetable oil acreage (Singh et al., 2013). It contributes 37% of global vegetable oil and 45% of edible oil worldwide with a market share of ~32% (Morcillo et al., 2013). Palm oil is used in food and in the chemical and biofuel industries.

It has been reported that conditions of drought and source-sink stress induce male inflorescences in undifferentiated inflorescence meristems in oil palm (Durand-Gasselín et al., 1999; Corley and Tinker, 2003; Adam et al., 2011). Stress is said to affect the reproductive process in oil palm according to stages of inflorescence development. Severe defoliation in plants leads to a significant decrease in the photosynthetic process, thereby limiting the production and accumulation of carbohydrate reserves (Simbo et al., 2013). Under conditions of sugar depletion, crops acclimate by modifying physiological and biochemical processes. Low water availability and low sugar supply to flower tissues cause excessive accumulation of reactive oxygen species (ROS), which induce flower abortion through programmed cell death

(PCD) (Foyer and Noctor, 2005). The impact of stress on the undifferentiated inflorescence meristem of oil palm situated at leaf axil -20 is the determination of the inflorescence sex, while the impact on postdifferentiated inflorescences may be inflorescence tissue death (Durand-Gasselín et al., 1999; Adam et al. 2011).

Plants respond to abiotic stress by altering the expression of thousands of genes involved in molecular, cellular, and biological processes (Chinnusamy et al., 2007). Most of these genes are regulatory in nature and thus their expression is time- and tissue-specific. Ajambang et al. (2015a) reported that the amount of total soluble sugar was reduced by 55% in leaves and 21% in inflorescence of oil palm trees 45 days after complete defoliation (45 DAD) stress. Thus, analyzing tissue-specific transcriptomes under stress at a specific moment in time can give us an understanding of the set of genes controlling specific biological processes and the dynamics of their expression patterns. Ajambang et al. (2015a) reported that the specific moment at which genes are differentially expressed in inflorescence meristem in response to complete defoliation is between the 30th and 60th day after defoliation. Hence, samples for differential gene expression were collected at

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45 DAD. According to Geiger et al. (1996) and Adam et al. (2005), research is needed to identify, characterize, and reveal the genetic and molecular mechanisms responsible for these regulatory processes. It would be interesting to identify and quantify the set of genes in the molecular, biochemical, and cellular processes responding to mechanical and abiotic stress in oil palm.

Molecular genetic techniques have been used to study the transcriptomes of different plant organs (Morcillo et al., 2013; Mohammadkhani et al., 2016). Recent advances in high-throughput next-generation sequencing have provided great potential for the production of large-scale transcriptomic data. Bourgis et al. (2011) and Tranbarger et al. (2011) used 454 pyrosequencing to produce transcriptomic sequences of different organs of oil palm. RNA-seq is the trending approach for gene expression studies. RNA-seq is the use of high-throughput sequencing-based technologies to investigate gene expression patterns in which transcriptome information can be derived for sequences of more than 100 base pairs. RNA-seq data originate directly from functional genomic items whose functions may be documented or undocumented. RNA-seq is more advantageous because of its lower costs, increasing transcript yields, and improved bioinformatics data mining, which makes it possible to obtain sequence information and differences in gene expression levels between tissues and elucidate gene network complexity by direct sequencing (Wolf et al., 2010).

The aim of this present study is to analyze global gene expression from a developing inflorescence of oil palm under complete defoliation stress conditions using RNA-seq transcriptome profiling to identify the set of genes expressed on oil palm inflorescences as a result of complete defoliation stress or carbohydrate deprivation and to quantify stressed inflorescence gene expressions and categorize their biological functions.

2. Materials and methods

2.1. Plant materials and experiment

Samples of 6 oil palm trees of La Me pisifera origin were used for this experiment. The experimental unit was a single tree, replicated three times. The control consisted of 3 trees that were not defoliated. Complete defoliation treatment involved the removal of all opened leaves of the oil palm as described by Durand-Gasselin et al. (1999). The trees were kept under complete defoliation stress for 45 days, after which tissue samples were collected for RNA isolation. It has been estimated that pisifera initiates response mechanisms to stress caused by a halt in the photosynthetic process due to complete defoliation, in the second month after stress treatment (Ajambang et al., 2015b). The trees were felled in the early hours of the day and were cut at the level of their crowns located around leaf

number +40. The leaf numbers were obtained by counting from leaf number 0, which is represented by the middle unopened leaf. Leaf number +1 was obtained by counting outwards to the left direction from leaf number 0. Leaf number -1 was obtained by counting inwards towards the right direction from leaf number 0. Leaf petioles were removed one after another inwards towards leaf 0 until leaf number -30. A sharp blade was used to dissect the meristem and carefully extract the inflorescence tissue located on leaf axil number +5 for total RNA.

2.2. RNA sample preparation

Total RNA was isolated from the 100 mg of tissues using the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). A NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the concentration and optical density of the RNA samples. RNAsstable (Biomatrix, San Diego, CA, USA) was used for the stabilization of the RNA in preparation for its transportation to Korea. Total RNA integrity (RIN) was checked using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). A minimum RIN value of 7.2 was used to select quality samples. The mRNA fragments were converted from 4 µg of total RNA samples prepared into cDNA using the TruSeq v2 RNA Sample Preparation Kit (Illumina Inc., San Diego, CA, USA). Poly-A enrichment was used to obtain mRNA from total RNA as described by Ajambang et al. (2015b). The mRNA was purified using poly-T oligo-linked magnetic beads in two rounds of purification. First-strand cDNA was synthesized using SuperScript II reverse transcriptase and random primers while DNA polymerase I was used to synthesize second-strand cDNA. DNA fragments containing adapter molecules on both ends were enriched using a PCR primer cocktail in a 15-cycle 96-well PCR reaction. The PCR products were cleaned up using AMPure XP beads and quantified using qPCR. Quality control was done on an Agilent Technologies 2100 Bioanalyzer using a DNA Agilent DNA-1000 chip. Clustering was done on the cBot system using TruSeq PE Cluster Kit V3 – HS (cBot) (Illumina Inc.). Sequencing was done to generate 100-bp PE reads on the Illumina HiSeq 2000 instrument (Illumina Inc.) at the Macrogen Next Generation Sequencing Division (Macrogen, Seoul, Korea). The cDNA multi FASTA file was aligned against the Arabidopsis Information Resource (TAIR10) using the Blast 2.2.25+ version (www.arabidopsis.org).

2.3. Quality control and analysis of sequencing results

Removal of low-quality reads was performed with Illumina Pipeline (CASSAVA) software v.1.8.2 (Illumina Inc.). Mapping of FASTQ data from control samples was performed with EG5_genes V2. gff3 for the oil palm genome (<http://www.genomsawit.mpob.gov.my>) using the RNA-seq reads alignment software TopHat2-

SE v1.3.3 for paired-end sequencing. Cufflinks v2.0.2 (Trapnell et al., 2010) was used to assemble the RNA-seq alignments generated by TopHat and then estimate their relative abundance as fragments per kilobase of exon per million fragments mapped (FPKM). Transcripts with zero FPKM were excluded from downstream analysis. The gtf files generated by cufflinks were merged into a single transcriptome annotation file, merge.gtf, using the software Cuffmerge. Expression analysis was performed using the software CuffDiff. A squared coefficient of variation plot was run to identify any cross-replicate variability in order to evaluate sample data quality.

Fold change (FC) (Tusher et al., 2001) was calculated by $\log_2FC = \text{normalized value of test} - \text{normalized value of control}$. Fold change was later converted to a linear value using the equation $FC = 2^{\log_2FC}$. When values of FC were between 0 and 1, FC was calculated by a negative reciprocal ($= -1/FC$) and this indicates that there was a downregulation of genes. When FC values were greater than 1, then FC was equal to the actual FC value. $|FC| \geq 2$ and $P < 0.05$ from an independent t-test were used to filter differentially expressed transcripts between samples. The

cut-off point for fold change was voluntarily designed to focus on genes showing strong differences between tissues.

Gene ontology (GO) was analyzed from TAIR10 (www.arabidopsis.org) and gene set enrichment analysis was performed with DAVID (Huang et al., 2009).

3. Results

3.1. Transcriptome sequencing and assembly

Global gene expression was done for both treated and control cDNA libraries. Sequencing of control libraries yielded 29,805,424 read counts of more than 3 billion bases. cDNA from treated samples produced 29,725,288 read counts and more than 3 billion bases.

3.2. DEG of genes at the inflorescence abortion stage

The volcano plot presented in Figure 1 gives the distribution of comparative gene expression between the defoliated and nondefoliated samples. The gene expression is defined by fold change and the P-value indicated by \log_2FC and $-\log_{10} P$ -value. Differentially expressed genes must have a P-value of < 0.05 and an absolute fold change value of ≥ 2 . The genes positioned out of the boundaries of the line $FC \geq$

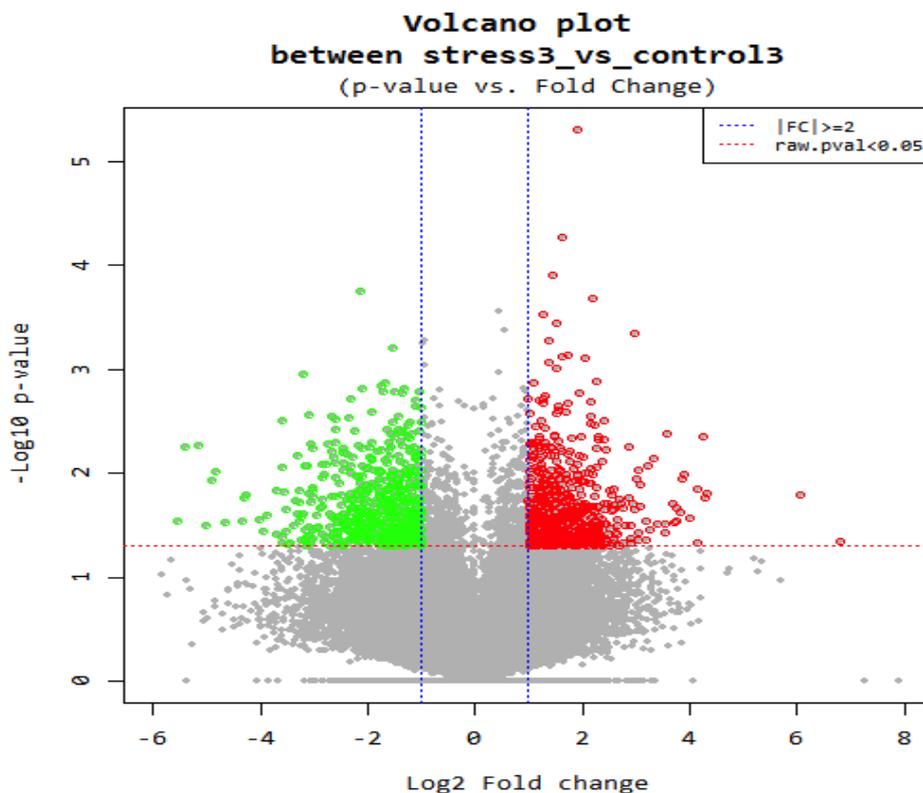


Figure 1. Volcano plot for DEGs between nondefoliated and defoliated samples.

The y-axis represents the level of significance of the expression change between samples measured on $-\log_{10} P$ -value (P -value = 0.05), while the x-axis represents the fold change (FC) of DEGs. DEGs located above the red line are significant based on the P-value while those below are not significant. The DEGs located between the two blue lines are not DEGs based on $|FC| \geq 2$.

2 are considered to be differentially expressed. The farther the distance is from the FC line, the higher the fold change is. Genes positioned from the left limit are downregulated while those at the right are upregulated.

Frequency of expression decreased with increasing expression levels, meaning that more genes were expressed at lower than at higher expression levels. This decreasing tendency was observed in both stressed and control tissues, as shown on Figure 2. The number of DEGs at $FC \geq 2$ without P-value restriction was 12,971, while it was reduced to 1214 at 372 at $FC \geq 2$ ($P = 0.05$) and $FC \geq 4$ ($P = 0.05$), respectively.

3.3. Functional classification based on gene ontology (GO)

Gene ontology is the classification of genes according to predefined criteria called GO terms that link all the genes available into that particular gene set. It is possible to retrieve the set of terms describing any gene and conversely to produce the set of genes annotated to any particular term. Classification is grouped in three categories: cellular processes (CP), biological process (BP), and molecular functions (MF). The 1214 DEGs at $FC \geq 2$ and P -value = 0.05 were categorized into these 3 categories. Fourteen functional groups were found for the BP categorization. It was observed that a high percentage of these genes were responsible for cellular and metabolic processes. About 15% of the genes were responsible for stress response while 13% were responsible for cell organization and developmental processes, as shown in Figure 3.

Gene set enrichment analysis was performed to verify that genes differentially expressed between the stress and

control tissues were functionally relevant to the processes related to the stress treatment. Gene set functional annotation clustering evaluated based on the enrichment score (ES) and the P-value produced a total of 157 clusters from the 1214 DEGs. Figure 4 gives the enrichment scores for the principal gene clusters.

The highly enriched clusters were composed of genes involved in cellular response to stress (ES = 3.2%), response to abiotic stress (ES = 3.12%), cell cycle (ES = 2.25%), stress response (ES = 2.18%), and response to carbohydrate stimulus (ES = 1.34%). The ES for these gene groups signifies their functional implications during response to complete defoliation stress in oil palm.

Generally, genes do not act alone; therefore, coexpression network analysis was used to find the functional networks in which both our downregulated and upregulated gene sets are involved. Table 1 shows the different functional networks for downregulated and upregulated genes.

4. Discussion

Downregulated genes were mostly involved in response to nutrient levels and starvation while upregulated genes were mostly responding to light intensity, ROS, and heat stress. We observe from Table 1 that the functions of upregulated genes are linked to nutrient source management while downregulated genes are linked to the sink. The upregulated DEGs are mostly photosynthetic genes working to produce more carbohydrates while the downregulated genes are working to efficiently use the limited amount of carbohydrates occasioned by stress.

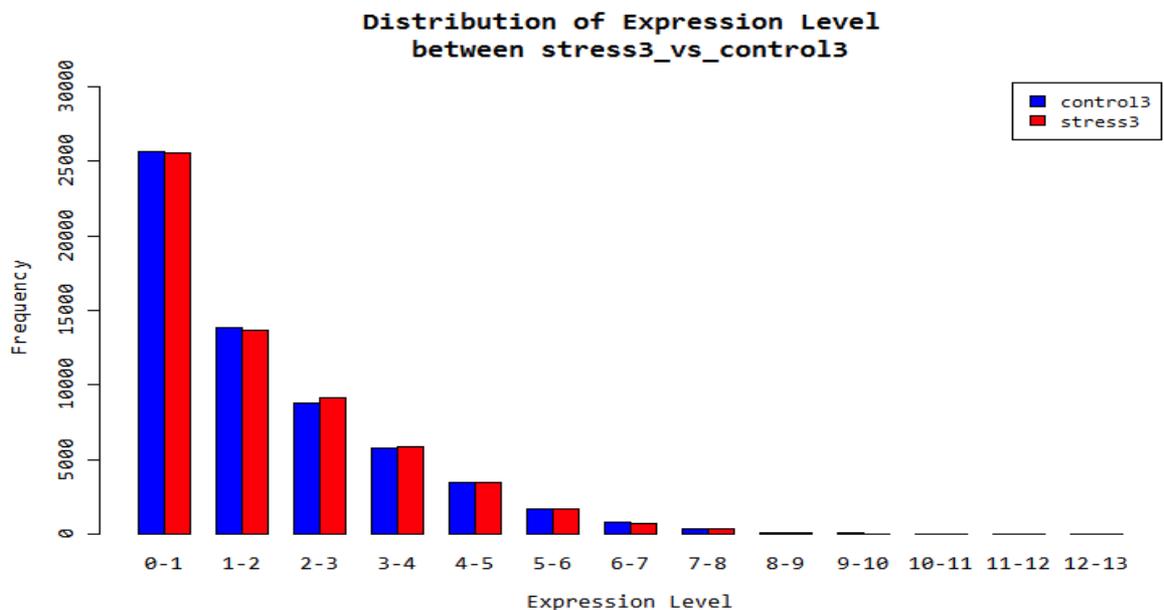


Figure 2. Distribution of gene expression level in stress and control tissues.

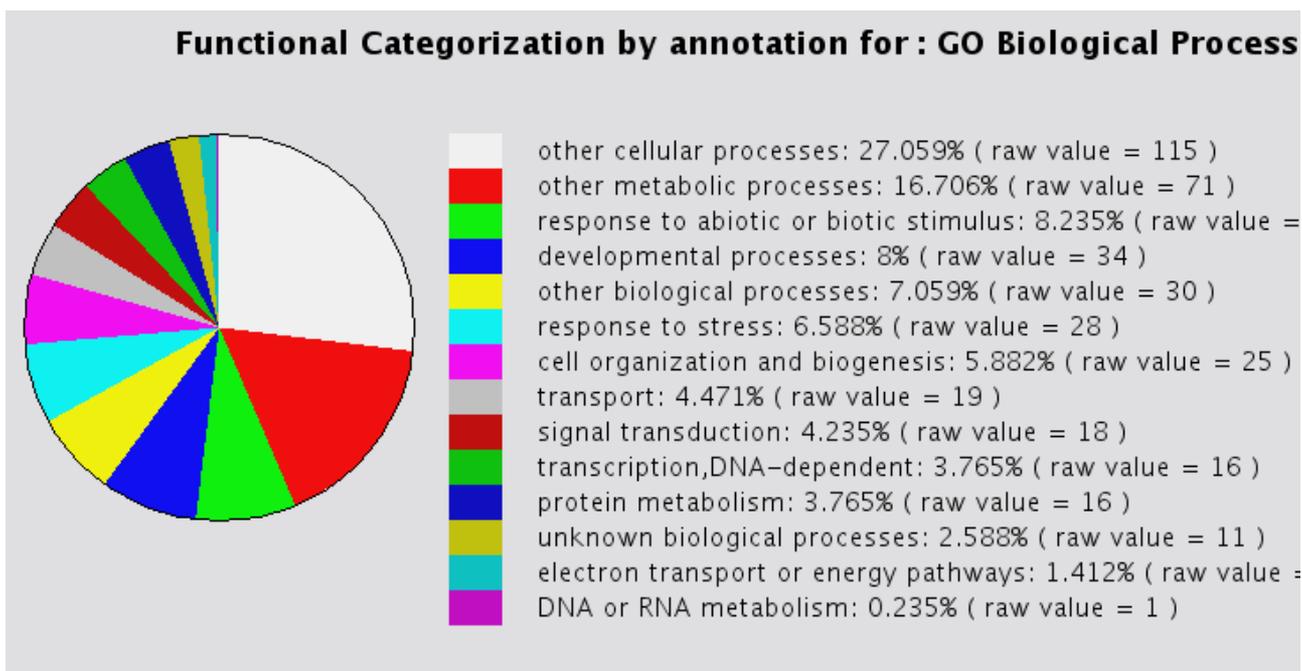


Figure 3. Functional categorization of DEGs based on biological processes.

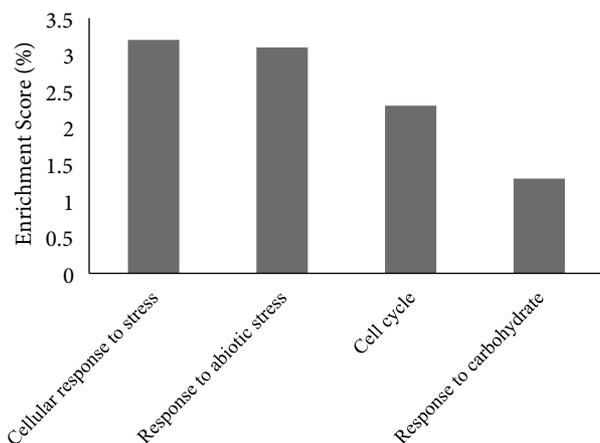


Figure 4. Enrichment scores for the principal gene clusters.

The downregulation of carbohydrate-processing genes in flower tissues during stress has been reported (McLaughlin and Boyer, 2004).

The ten top DEGs were selected based on their FC and P-values. The functions of these genes were obtained from TAIR. The majority of these genes are related to stress response and a few are senescence related genes or flowering and transcription activation genes. Genes that are downregulated carry a negative sign in the prefix while genes that are upregulated are positive integers. In Table 2, the DEGs are based on the experimental defoliated inflorescence tissue against nondefoliated inflorescence

tissue. An FC with negative sign indicates that the gene was highly expressed in the nondefoliated tissues $|FC|$ times compared to the defoliated tissues, where $|FC|$ is the absolute value of the fold change.

4.1. Description of the top ten DEGs and their biological functions

Genetic analyses have shown that family members of nudix hydrolase derivatives (*NUDT*) regulate signal intermediaries and toxicity levels in cells (Bartch et al., 2006). The *NUDT7* protein was identified as a negative regulator of defense response and its loss of function makes plants hyperresponsive to inciting agents (Ge and Xia, 2008). The presence of *NUDT7* in tissues causes growth retardation, necrotic lesions, and cell death in *Arabidopsis* (Bartch et al., 2006). The same study shows that *NUDT7* mutants exhibited spontaneous death of cells in 2-, 3-, and 4-week-old plants. In our study, the *NUDT7* gene was highly downregulated at ~ 30 times in the stressed tissues as opposed to normal tissues. This downregulation may lead to growth retardation and eventually cell death, causing abortion of the tissues under stress. This PCD process is further supported by the upregulation of ROS in stressed tissues.

AT4G35750 encodes an *SEC14* cytosolic factor family protein that has been associated with regulation of signaling in response to hyperosmotic stress in plants through membrane trafficking and cytoskeleton dynamics (Mousley et al., 2007). *SEC14* is involved in lipid signaling-mediated plant immune response and its loss of function

Table 1. Functional networks involving downregulated and upregulated DEGs.

Downregulated DEGs	Upregulated DEGs
Cellular response to P starvation	Response to high light intensity
Response to nutrient levels	Response to hydrogen peroxide/ROS
Cellular response to external stimulus	Response to heat
CHO derivative biosynthesis	Response to light intensity

Table 2. Ten top DEGs based on FC and P-value.

Gene ID	FC	Function
AT4G37820	110	Unknown protein
AT4G10250 (<i>HSP</i>)	67	Abiotic stress response
AT5G59720	20	Abiotic stress response
AT4G32570 (<i>TIFY8</i>)	19	Activator of JA
AT1G52520 (<i>FRS6</i>)	17	Light control of flowering
AT3G02040	-47	Senescence-related gene
AT1G61667 (<i>DUF538</i>)	-42	Response to stress
AT1G12630	-35	Transcription activation
AT4G35750 (<i>SEC14</i>)	-32	Senescence
AT1G01670 (<i>NUDT7</i>)	-30	Cell death

led to jasmonic acid (JA) accumulation in tobacco (Kiba et al., 2014). JA has been associated with PCD and tissue senescence in plants (Reinbothe et al., 2009). JA was responsible for male flower development and pistil (female flower) abortion in maize through a tassel seed-mediated cell death process (Acosta et al., 2009). Sample tissues in this study originated from female inflorescences; thus, the high accumulation of JA may be responsible for the abortion of inflorescences located in leaf axil +5 in oil palm (Durand-Gasselin et al., 1999). On the contrary, the gene *AT4G32570* encoding the *TIFY* domain protein 8 that is upregulated in our stress tissues is a JASMONATE ZIM (*JAZ*) domain mediator, which plays a role in JA repression. Chung and Howe (2009) reported that the *TIFY* motif suppressed *JAZ* signaling, thereby activating JA accumulation in Arabidopsis. Therefore, an upregulation of genes that will positively regulate repressors of JA signaling and a downregulation of genes whose loss of function activates JA accumulation are observed.

Expression of the *DUF538* domain has been related to stress response in plants. The *DUF538* protein was shown to increase redox enzyme activities including catalase,

peroxidase, and poly-phenol oxidase (Gholizadeh, 2011). The gene *AT1G52520* encodes the far-red impaired response (*FARI*, *FRS6*) that regulates flowering time in response to various biotic and abiotic signals. *FRS6* mutants acted as positive regulators in the phytochrome b (*phyB*) signaling pathway, which plays an inhibitory role in floral initiation (Lin and Wang, 2004). In our experiment, *FRS6* was upregulated in defoliated tree tissues, indicating that complete defoliation stress favors male inflorescence induction in oil palm. Carbohydrate starvation has been responsible for PCD in plants. Min et al. (2013) reported that altered carbohydrate metabolism led to PCD that further induced anther sterility in cotton. Some studies have established a link between carbohydrate concentration and ethylene activity (Imanishi et al., 1994; Iglesias et al., 2006). Ethylene has been identified as one of the hormones that have profound effects on growth and development including PCD (Fluhr and Mattoo, 1996; Young et al., 1997). Ethylene induced PCD in response to pathogen attack (Greenberg et al., 1994) and formed lysogenic aerenchyma in maize roots in response to hypoxic conditions (He et al., 1996).

Through RNA-seq carried out on samples extracted 45 DAD, we produced the set of genes responding to complete defoliation stress and also showed that genes were expressed differentially between stress and control inflorescence tissues. Upregulated DEGs were mostly photosynthetic genes working to produce more carbohydrates while downregulated genes were carbohydrate metabolism genes, working to efficiently use the limited amount of carbohydrates. Gene enrichment has given evidence for cross-talk between stress response pathways and cell death in oil palm inflorescence tissues under stress. The accumulation of JA and the

downregulation of *NUDT7* genes may be the principal causes of young inflorescence abortion during stress. This is the first report of transcriptome analysis of a developing oil palm inflorescence collected at the internal stages of leaf development.

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