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Transcriptome Analyses of Tapping Panel Dryness (TPD) in *Hevea brasiliensis* Treated with Latex Stimulant RRIM HYDROBEST™

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ABSTRACT

The problem of tapping panel dryness (TPD) that renders the rubber tree non-yielding has been in the industry for over 100 years. Many studies have been done on TPD, however, the exact mechanisms remain speculative until today, while a definitive cure for the disorder remains unavailable. Recently, a newly developed latex stimulant, RRIM HYDROBEST™ has been shown not only to increase latex production but also to reduce the TPD incidence. In order to reveal both TPD and also RRIM HYDROBEST™ mechanisms, transcriptome of latex between three groups (healthy, TPD, and TPD with RRIM HYDROBEST™) were obtained and compared. The differential expression analysis found 20, 678 and 577 statistically significant differentially expressed genes (DEGs) when comparing TPD versus Control (Comp1), TPD treated with RRIM HYDROBEST™ versus Control (Comp2), TPD with RRIM HYDROBEST™ versus TPD (Comp3), respectively. The enrichment tests retrieved a total of 119, 2,264, and 2,179 GO terms and 0, 24, and 24 pathways which might be associated with the three transcriptome profilings, Comp1, Comp2, and Comp3, respectively. Most significantly enriched KEGG pathways included alanine, aspartate and glutamate metabolism, taurine and hypotaurine metabolism, nitrogen metabolism, flavone and flavanol biosynthesis, and glycosaminoglycan degradation. The transcriptional responses of latex rubber tree to the TPD and RRIM HYDROBEST™ were determined and a large number of DEGs were characterised, providing important clues for further elucidation of the mechanisms of TPD stress response and RRIM HYDROBEST™ effect in rubber tree.

Keywords: Latex, oxidative stress, tapping panel dryness, transcriptome

INTRODUCTION

Hevea brasiliensis is a major rubber producing plants. Even though rubber can be produced synthetically, natural rubber from *H. brasiliensis* is still irreplaceable. The natural rubber has unique properties which includes resilience, elasticity, abrasion and impact resistance, efficient heat dispersion, and malleability at cold temperatures, which cannot be matched by synthetic rubber (Cornish, 2001). *H. brasiliensis* is not the only plant that can produce rubber in the world. About 2500 plants from various taxa distributed throughout the plant kingdom have been identified to produce rubber. However, these plants have a very low yield of rubber or low molecular weight of polymer (van Beilen and Poirier, 2007). Consequently, *H. brasiliensis* becomes the only rubber-producing plant species that have been cultivated commercially for natural rubber.

Due to the global dependence on *H. brasiliensis* as the source of natural rubber, it is important to optimise the health status of the trees. There is one physiological disorder, known as Tapping Panel Dryness (TPD), that ubiquitously affects *H. brasiliensis* resulting in 12 to 20% reduction in dry rubber production and some cases of high TPD incidence can cause reduction of more than 50% (Chen et al., 2003). The trees that have been affected with TPD experience cessation latex flow when tapped. TPD has been studied since

the start of natural rubber industry as early as 1920s but the problem has yet to be resolved. This is due to the fact that the mechanism of this physiological disorder in *H. brasiliensis* is not fully understood. Recent studies have indicated that TPD is a physiological disorder resulting from abiotic stress. In earlier studies of TPD, the involvement of a pathogen in the occurrence of TPD seems to be a possibility, but the results were far from being conclusive (Peries and Brohier, 1965; Lim et al., 1973; Ramachandran et al., 2000). *H. brasiliensis* trees that succumb to TPD are mostly trees that have been tapped and stimulated with ethylene excessively. TPD can be categorised into two types, which are a reversible tapping cut dryness without any visible sign of bark necrosis, and an irreversible bark necrosis or also known as brown bast (Jacob et al., 1994).

Although some progress has been made with regards to understanding TPD, the underlying molecular mechanisms of TPD remain largely unknown. In this respect, a prerequisite is to identify TPD-related genes and analyse their expression patterns and functions. In this study, the availability of *H. brasiliensis* transcriptomic and genomic resources made it possible to identify all the different genes acting in the ethylene signalling pathway and characterise their implications during development, and in response to TPD. The latex tissue of RRIM 3001 was used for identifying the responsible genes and the expression patterns of these genes made possible by RNA-sequencing deployed in this study. In addition, the effect of the newly developed RRIM HYDROBEST™ on *H. brasiliensis* was studied at the molecular level as it had been shown to reduce TPD incidence (Abdul Ghaffar et al., 2018). By having this information, thorough mechanism of TPD incidence and the role of RRIM HYDROBEST™ in the mechanism can be elucidated. In the near future, these results can be used as a platform for developing an effective treatment for TPD.

MATERIALS AND METHODS

Plant materials and RNA extraction

RRIM 3001 was planted at the experimental field 109, RRI Experimental Station (RRIES) Pelepah, Kota Tinggi, Johor in 2008. The newly formulated latex stimulation used was RRIM HYDROBEST™ with a concentration of 2.5% once a month. In this study, the trees with normal latex flow were considered as "healthy" trees, whereas trees with partial flow were referred to as TPD trees. The latex sample was collected from three groups of trees namely healthy trees without RRIM HYDROBEST™ (Control), TPD-affected trees without RRIM HYDROBEST™, and TPD-affected trees with RRIM HYDROBEST™. Each group of trees had three biological replicates with one tree per replicate. For RNA extraction, an extraction buffer (300 mM LiCl, 10 mM disodium salt EDTA, 10% SDS and 100 mM Tris) was added with the same amount of latex and was then kept on ice. Once arrived in the lab, the same amount of TRIzol was added to the mixture and total RNA was extracted according to the manufacturer's instructions (Invitrogen Life Technologies RNA, UK).

Library preparation and sequencing

Total RNA of each sample was quantified and the quality determined using Agilent 2100 Bioanalyzer (Agilent Technologies) and NanoDrop (Thermo Fisher Scientific Inc.). Total RNA (1 μg) with RNA Integrity Number (RIN) value above 7 was used for library preparation. Next generation sequencing library preparations were constructed according to the manufacturer's protocol (NEBNext® Ultra™ RNA Library Prep Kit for Illumina®). Paired-end sequencing was performed using Illumina HiSeqX platform (Illumina, San Diego, CA, USA) for three independent biological replicates.

Quality assessment and pre-processing

Quality of raw sequencing reads were assessed by FastQC v0.11.8 (Andrews, 2010). Pre-processing of the data were performed using Trimmomatic v0.38 (Bolger et al., 2014) with the parameters comprising

ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:20 TRAILING:20 SLIDINGWIND OW:8:20 MINLEN:50.

Read mapping

Clean reads of each sample were mapped to the reference genome (*H. brasiliensis*, RefSeq assembly accession: GCF_001654055.1) using HISAT2 v2.1.0 (Kim et al., 2015) with the following parameters comprising minimum intron length = 50, maximum intron length = 15,000, and reports alignments tailored specifically for cufflinks.

Expression and differential expression

Analyses of reference gene expression and differential expression were carried out using Cufflinks v2.2.1 (Trapnell et al., 2010; Roberts et al., 2011) and Cuffdiff v2.2.1 (Trapnell et al., 2010, 2013) with the following parameters namely maximum fragments allowed in a bundle before skipping = 5,000,000, use 'rescue method' for multi-reads (more accurate), and use bias correction - reference fasta required. The expression level of each gene was expressed as Fragments Per Kilobase of gene per Million fragments mapped (FPKM). Differentially expressed genes were defined as genes that are measured to be at least 4-fold different in expression level (log2 fold-change $\leq$ -2 or log2 fold-change $\geq$ 2) with false discovery rate less than 5% (q-value $\leq$0.05). In addition, genes of which the expression detected only in one condition with q-value $\leq$0.05 were also considered differentially expressed.

Gene ontology (GO) and pathway enrichment analysis

Differentially expressed genes were submitted for enrichment analysis against full reference genes. GO enrichment analysis was performed using Blast2GO v5.2.5 (Conesa et al., 2005) while pathway enrichment analysis was conducted using Fisher's exact test function in R v3.0.2.

RESULTS AND DISCUSSION

Latex transcriptomes sequencing and characterisation

Three cDNA libraries namely control (healthy), TPD, and TPD with RRIM HYDROBEST™ with three replicates each were sequenced using Illumina HiSeqX system. The total throughput for all nine transcriptome datasets were ~455 million raw reads or ~68.2 Gb of data with a minimum 6.4Gb data per sample. Sequencing reads were pre-processed to obtain high-quality data for further analysis. Quality assessment of sequencing reads were carried out on each dataset before and after data pre- processing. More than 92% of sequencing data were retained after quality filtering process, indicating sequencing was performed well (Table 1). Paired clean reads were aligned to reference genome of *H. brasiliensis* cultivar Reyan7-33-97 (Tang et al., 2016). Approximately 95% of reads from each sample were mapped to the reference genome with unique mapping rate achieving around 88% (Table 1).

Clean reads mapped to genes annotated in reference genome were quantified and normalised to gene expression unit termed as FPKM. Genes with FPKM = 0 were considered to be not expressed, while genes with FPKM more than 0 were considered to be expressed genes. An average of approximately 59% of the 42,489 reference genes tested were found to be expressed in each sample.

Identification of differentially expressed genes (DEGs)

Differential expression analyses among sample groups were conducted in pairwise (in "Test vs. Control") design. In each comparison, sample Group A served as control set while sample Group B served as test set. In brief, 20 to 678 DEGs were observed in each comparison (the top DEGs are shown in Table 2). The

differential expression analysis results were summarised in Figure 1. TPD-related genes (Comp1) recorded the lowest number of DEGs with only 20 DEGs. Twelve of them were upregulated and eight were downregulated. The effect of RRIM HYDROBEST™ on DEGs was markedly higher in both comparisons (Comp2 and Comp3).

Table 1. Overview of transcriptome sequencing and mapping

Sample	Raw reads numbers	Clean read numbers (%)	Clean bases	Q20 (%)	Mapped reads (%)	Uniquely mapped reads (%)	Multi-mapped reads (%)
Control	44,869,236	42,394,557 (94.48)	6,730,385,400	97.30	40,333,148 (95.14)	37,560,259 (88.59)	2,772,889 (6.55)
TPD	58,427,340	55,577,080 (95.12)	8,764,101,000	97.67	52,463,542.6 (94.26)	49,149,584 (88.26)	3,313,958 (6.00)
TPD with HYDROBEST ™	48,337,109	45,760,932 (94.67)	7,250,566,400	97.37	43,301,983 (94.63)	40,288,980 (88.04)	3,013,003 (6.59)

Table 2. List of Top DEGs

Treatments	Upregulated	Downregulated
TPD vs Control	Peroxisomal membrane protein 11C, ER lumen protein-retaining receptor A, probable calcium-binding protein CML45, ycf20-like protein, ankyrin repeat-containing protein NPR4-like	uncharacterized protein, nudix hydrolase 26 chloroplastic-like, Disease resistance protein RFL1-like, long non-coding RNA, E3 ubiquitin-protein ligase MARCH2-like
RRIM HYDROBEST ™ vs Control	aquaporin TIP1-1-like, aquaporin TIP1-3-like, major latex allergen Hev b 5, probable WRKY transcription factor 40, carbonic anhydrase 2-like	1-deoxy-D-xylulose 5-phosphate synthase, acetyl coenzyme A acetyltransferase, hydroxymethylglutaryl-CoA synthase-like, transcription factor bHLH25-like, esterase
RRIM HYDROBEST ™ vs Control	glutathione S-transferase, geranylgeranyl diphosphate reductase, TPR repeat-containing thioredoxin TTL1-like, carbonic anhydrase 2-like, 17.3 kDa class I heat shock protein-like	oxidase, peroxidase 42-like, peroxidase 64-like, transcription factor bHLH25-like, serine/threonine-protein kinase AtPK2/AtPK19-like

The mechanism of TPD cannot be clearly elucidated as the number of DEGs obtained from this study were small. Furthermore, the samples were collected at the early stage of TPD with less than 25% dry along the tapping panel when the rubber tree response to TPD might not as robust as later stages. However, some DEGs obtained from the comparison between TPD and Control samples indicated that reactive oxygen species (ROS) could be involved. These DEGs have not yet being characterised but their orthologues and gene family from different species that have been studied could provide useful information about them. For example, peroxisomal membrane protein 11C was upregulated in the TPD affected trees, and its orthologue in *Arabidopsis thaliana* was part of the organelle that produced ROS under stress environments (Su et al., 2019). Other upregulated genes such as ER lumen protein-retaining receptor A-encoding gene and calcium-binding protein CML45-encoding gene might also be involved in ROS and

stress response as indicated by their orthologues in *A. thaliana* (Numers et al., 2010; Marmiroli et al., 2015). In the future, these DEGs should be further characterised to have better understanding of early TPD response in rubber trees. For advanced stage of TPD, recent studies have indicated that reactive oxygen species (ROS) could play vital roles in TPD (Putranto et al., 2015; Zhang, Leclercq and Montoro, 2017; Zhang et al., 2019).

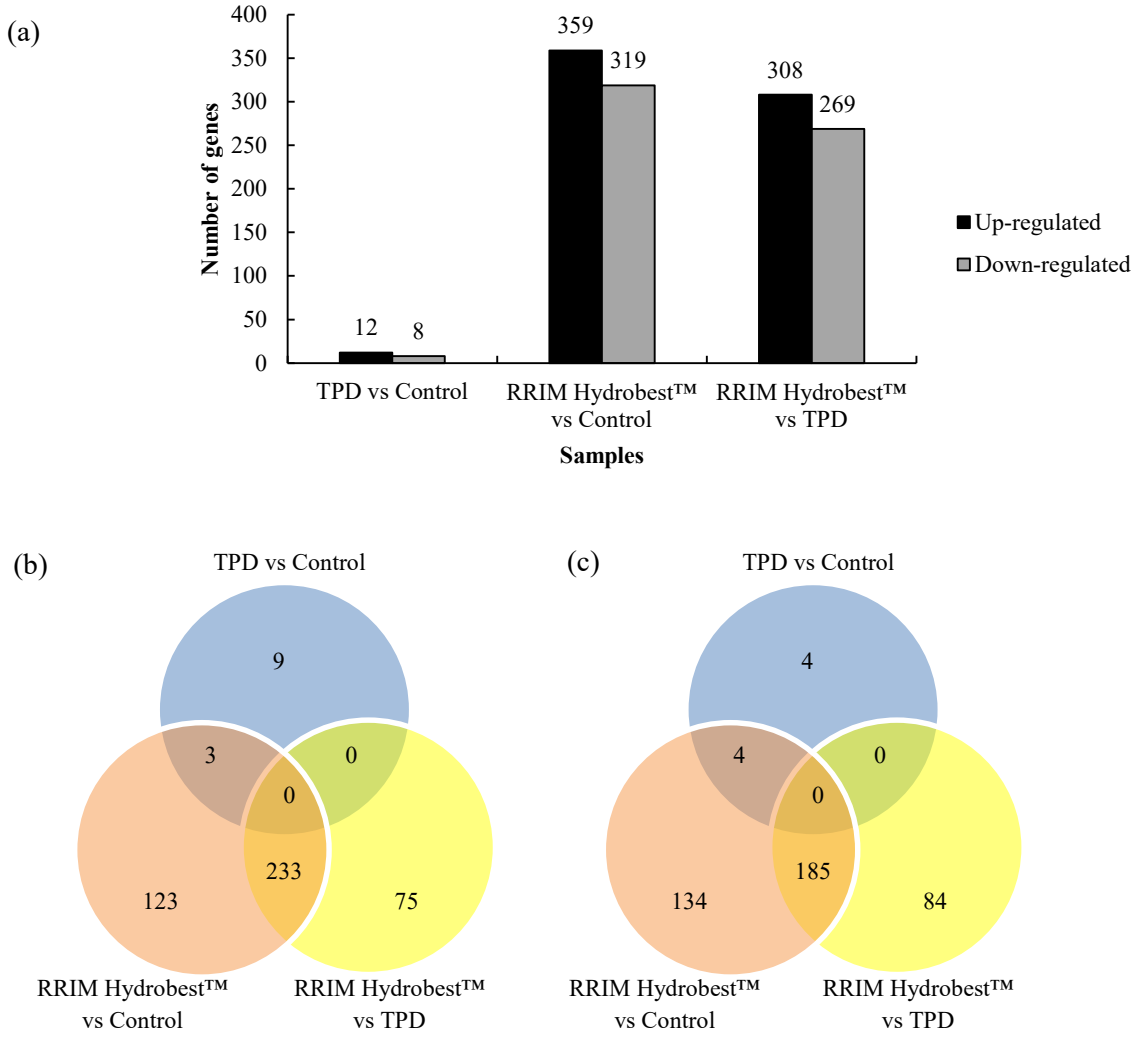

Figure 1. The statistics of DEGs obtained in this study. Numbers of DEGs between TPD and Control, between RRIM HYDROBEST™ and Control, and between RRIM HYDROBEST™ and TPD (a). Venn diagram of the numbers of upregulated (b) and downregulated (c) DEGs between TPD and Control, between RRIM HYDROBEST™ and Control, and between RRIM HYDROBEST™ and TPD.

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The ability of RRIM HYDROBEST™ to enhance latex yield could be by prolonging latex flow. This has been indicated by the upregulation of several genes related to the laticifer water balance and the inhibition of latex coagulation upon the treatment with RRIM HYDROBEST™. Sufficient water supply is vital in each tapping as 60 to 70% of latex is water (Jacob et al., 1989) and the fact that the mature laticiferous vessel rings are devoid of functional plasmodesmata connections (De Fay et al., 1989), the transport of water into the mature laticiferous vessel requires aquoporins. The results showed that two aquaporin genes (HbTIP1-1 and HbTIP1-3) from a subfamily known as tonoplast intrinsic proteins (TIPs) were upregulated with RRIM HYDROBEST™ treatment suggesting the increase of latex yield could be due to the additional supply of water. In addition, HbTIP1-1 was also reported to be upregulated upon treatment with Ethrel, a different kind of latex stimulant (Tungngoen et al., 2009). In addition, the prolonged latex flow enhanced by RRIM HYDROBEST could be due to the inhibition of latex coagulation as indicated by the upregulation of major latex allergen Hev b 5. Although the role of Hev b 5 is unclear, another type of latex allergen known as Hev b 7 has been shown to act as an antagonist of rubber particle aggregating factor (Shi et al., 2016). The characterisation of Hev b 5 should be done in the future to confirm its involvement in prolonging latex flow.

Although it was assumed that the increased latex yield when treated with RRIM HYDROBEST™ was due to the increase in latex regeneration, none of the biosynthesis related genes were upregulated. In contrast, some of the genes involved in producing isopentenyl pyrophosphate (IPP), the precursor for rubber biosynthesis (Chow et al., 2012), such as 1-deoxy-D-xylulose 5-phosphate synthase, acetyl coenzyme A acetyltransferase and hydroxymethylglutaryl-CoA synthase-like, were found to be downregulated. This could be the reason that the dryness incidence when using RRIM HYDROBEST™ was low compared to the other type of latex stimulants as the tree was not forced to increase its capacity to over produce rubber.

In addition to not overstimulate the rubber tree to produce more rubber, RRIM HYDROBEST™ aided the rubber tree in reducing TPD by maintaining a balanced ROS content in the cell. Unlike the treatment using ethephon where the genes related to ROS scavenging were downregulated (Nie et al., 2016), the treatment with RRIM HYDROBEST™ showed otherwise. The frequent environmental stress imposed by tapping can increase the intracellular level of ROS and thus it is important to counter this by increasing activity of antioxidants and ROS scavenging enzymes with the use of latex stimulant. In this study, glutathione S-transferase, geranylgeranyl diphosphate reductase, and TPR repeat-containing thioredoxin TTL1-like genes, which have roles in ROS detoxification (Balabaskaran and Muniandy, 1984; Chow et al., 2007; Wang et al., 2015), were upregulated with the treatment of RRIM HYDROBEST™. Another way how the ROS level in the cell is kept low by RRIM HYDROBEST™ could be by reducing the ROS production as indicated by the downregulation of several related genes such as polyphenol oxidase, peroxidase 42-like and peroxidase 64-like (Wititsuwannakul et al., 1997; Wang et al., 2015).

Functional classification and characterisation of DEGs

Based on sequence identity, GO terms associated to DEGs were tested using enrichment analysis. Numbers of GO terms achieving P-value ≤ 0.1 were summarised in Figure 2. TPD related-genes were mostly associated with biological process followed by cellular component and molecular function. The same observation was obtained for the comparison related to RRIM HYDROBEST™. For TPD related genes, the prominent GO terms of biological process, cellular component and molecular function were cellular process, cell part and binding, respectively (Figure 3). For the DEGs related to the response to

HYDROBEST™, in the GO terms of "biological process", the categories of "cellular process" and "biological regulation" were prominent. The categories of "cell" and "organelle" were dominant in the GO terms of "cellular component". In the GO terms of "molecular function", a high percentage of DEGs were associated with "catalytic activity" and "binding".

To further understand the functions of DEGs, we mapped all DEGs to KEGG pathways and compared them with the whole-transcriptome background, with the goal of searching for genes involved in metabolic or signal transduction pathways that were significantly enriched. Pathways achieving P-value ≤ 0.1 were summarised in Figures 4 and 5. TPD-related genes were not enriched to any KEGG pathways. This could be due to a small number of DEGs that cannot be mapped to KEGG pathways. The use of RRIM HYDROBEST™ mostly affected the pathways related to amino acid metabolisms and synthesis of several classes of isoprenoids. The upregulated DEGs were enriched in the pathways related to amino acid metabolisms, while the downregulated DEGs were enriched in the pathways related to synthesis of several classes of isoprenoids.

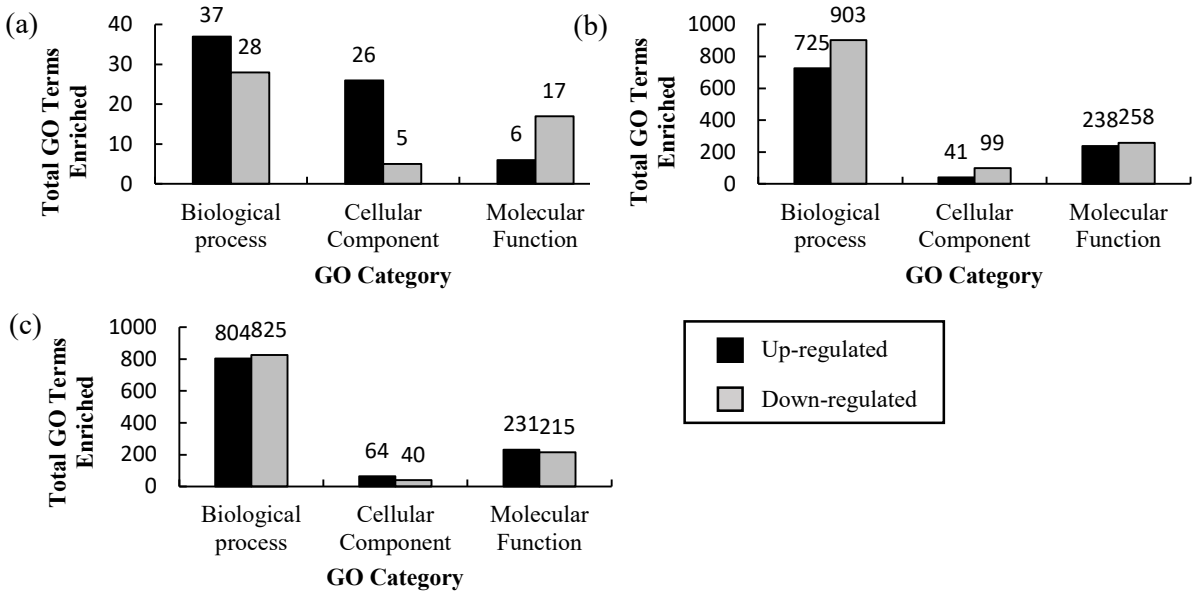
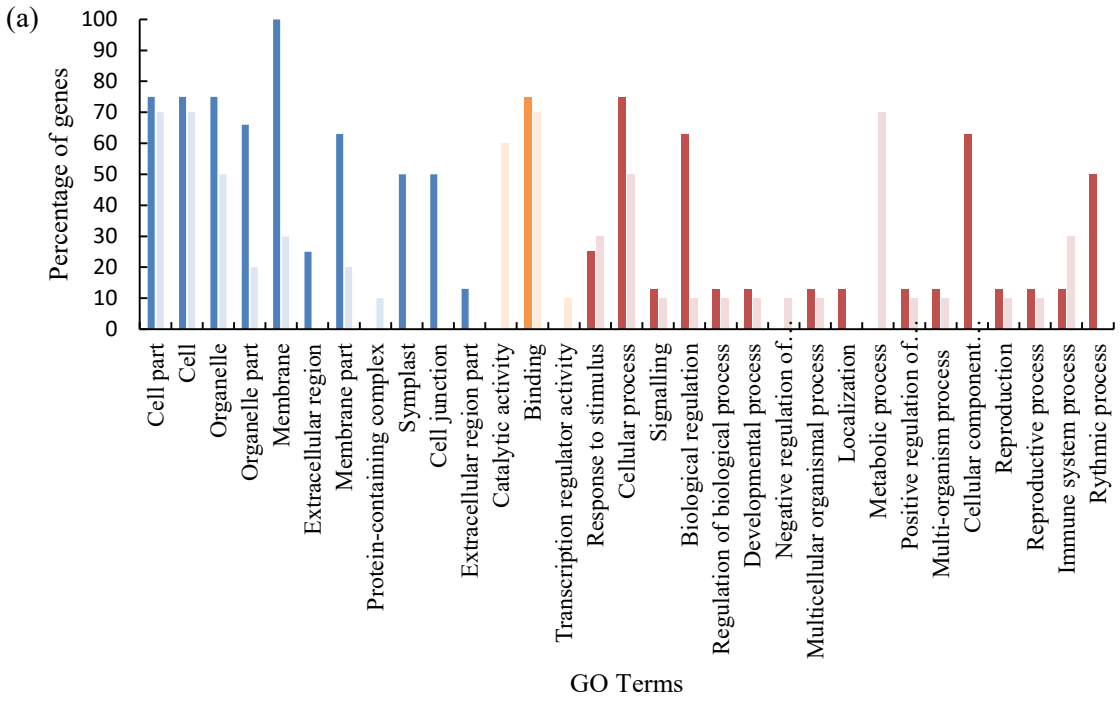
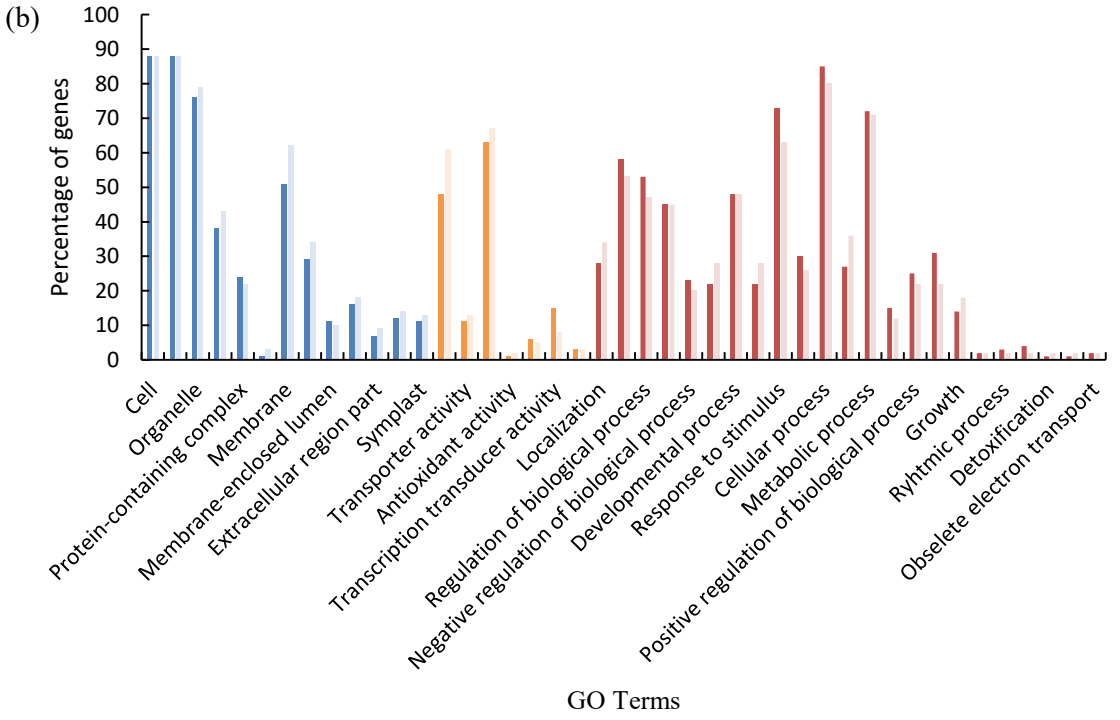


Figure 2. Number of enriched GO terms in DEGs between TPD and Control (a), between RRIM HYDROBEST™ and Control (b), and between RRIM HYDROBEST™ and TPD (c).


(a)
Percentage of genes
100
90
80
70
60
50
40
30
20
10
0
Cell part
Cell
Organelle
Organelle part
Membrane
Extracellular region
Membrane part
Protein-containing complex
Symplast
Cell junction
Extracellular region part
Catalytic activity
Binding
Transcription regulator activity
Response to stimulus
Cellular process
Signalling
Biological regulation
Regulation of biological process
Developmental process
Negative regulation of...
Multicellular organismal process
Localization
Metabolic process
Positive regulation of...
Multi-organism process
Cellular component...
Reproduction
Reproductive process
Immune system process
Rythmic process
GO Terms


(b)
Percentage of genes
100
90
80
70
60
50
40
30
20
10
0
Cell
Organelle
Protein-containing complex
Membrane
Membrane-enclosed lumen
Extracellular region part
Symplast
Transporter activity
Antioxidant activity
Transcription transducer activity
Localization
Regulation of biological process
Negative regulation of biological process
Developmental process
Response to stimulus
Cellular process
Metabolic process
Positive regulation of biological process
Growth
Ryhtmic process
Detoxification
Obselete electron transport
GO Terms

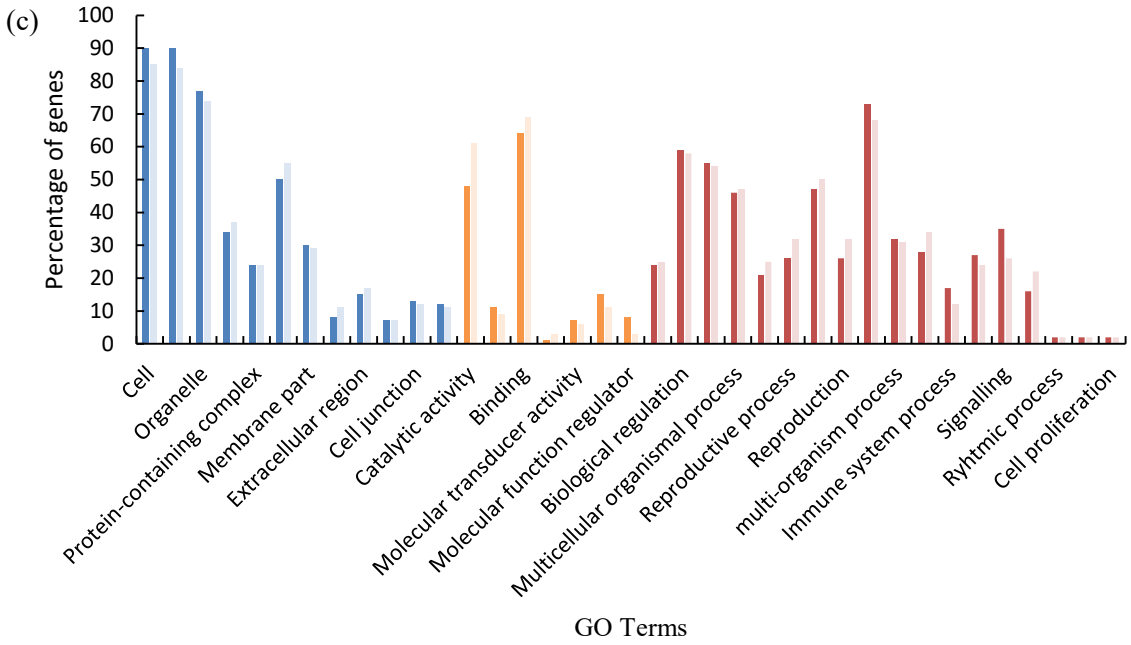


Figure 3. GO classification of the annotated DEGs between TPD and Control (a), between RRIM HYDROBEST™ and Control (b), and between RRIM HYDROBEST™ and TPD (c). The biological process, the cellular component and the molecular function are indicated by blue, orange and red respectively. The brighter colour represented the upregulated GO terms while the less bright colour represented the downregulated GO terms.

RRIM HYDROBEST™, similar to any other latex stimulants, contains ethephon that have been shown to increase latex yield in *H. brasiliensis*. Ethephon is an ethylene generator that increases latex yield by increasing latex regeneration and latex flow (Liu et al., 2016). However, there is an additional advantage of using RRIM HYDROBEST™ as highlighted in this study. The KEGG pathways analysis showed that the biosynthesis of other class of isoprenoids such as sesquiterpenoid and triterpenoid were downregulated when RRIM HYDROBEST™ was applied. This might indicate that the availability of isopentenyl diphosphate (IPP), which was a common intermediate for numerous isoprenoids, might be restricted to rubber biosynthesis only, which resulted in higher rubber production. Natural rubber is one type of isoprenoids and its biosynthesis requires IPP as the building block (Chow et al., 2012). To our knowledge, this effect can only be found with the application of RRIM HYDROBEST™ not ethephon. Other than ethephon, RRIM HYDROBEST™ consists of different chemicals that cannot be revealed due to the trade secret. These unclassified active ingredients could cause the effects mentioned earlier.

Other than increasing latex yield, RRIM HYDROBEST™ has been reported to reduce TPD incidence (Abdul Ghaffar et al., 2018). This can be associated with the roles of amino acid as many KEGG pathways related to amino acid metabolisms were upregulated. As being established earlier, TPD is caused by the excessive level of ROS. Thus, amino acids are important components of antioxidant systems in plants as they could reduce free radicals and osmoprotection (Gill and Tuteja, 2010; Rennenberg and Herschbach, 2014). Amino acids also can play a key role in signaling stress response and secondary metabolism in plants (Hildebrandt et al., 2015).

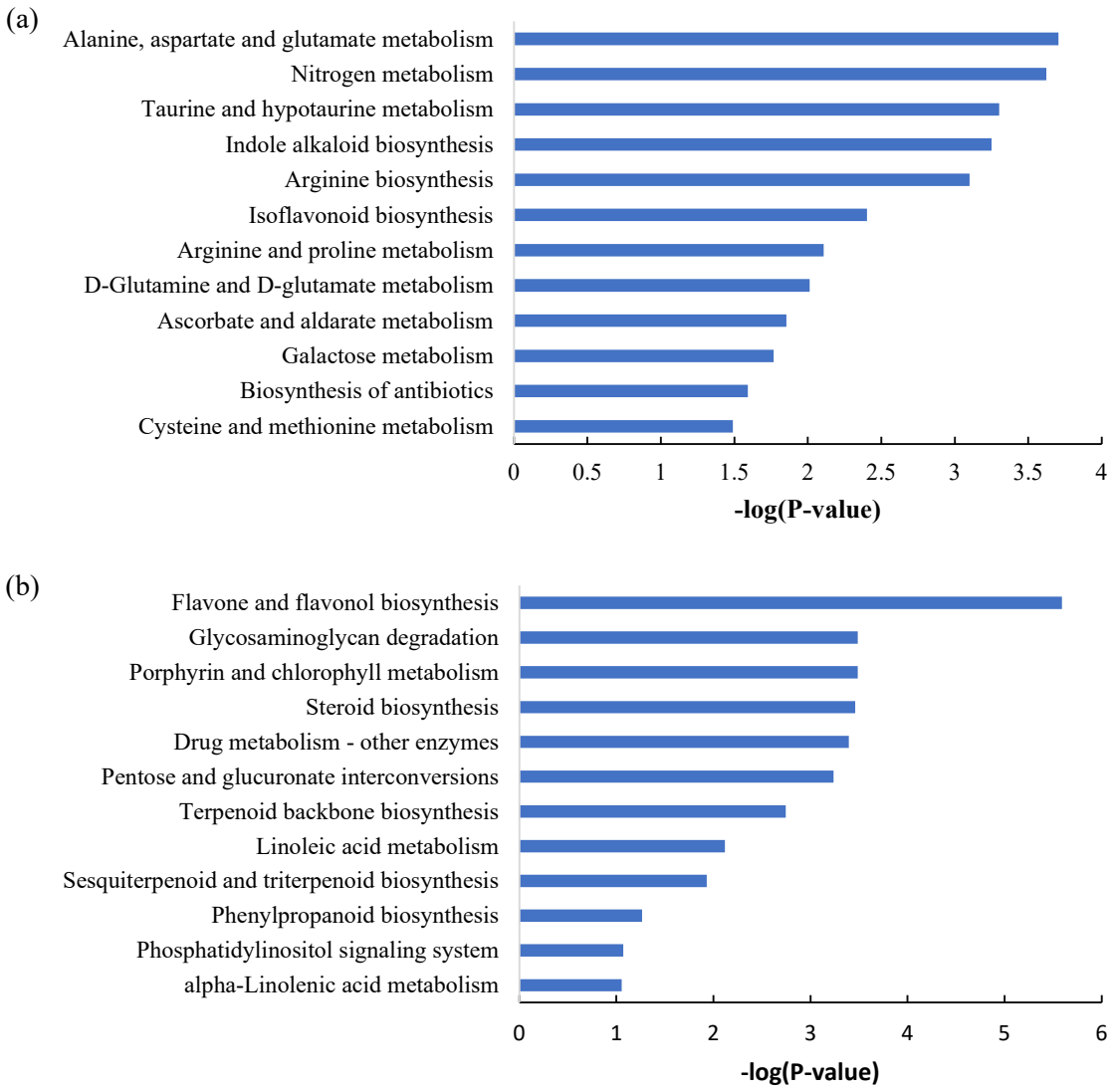


Figure 4. Pathways enriched in upregulated genes (a) and downregulated genes (b) of comparison between RRIM HYDROBEST™ and Control.

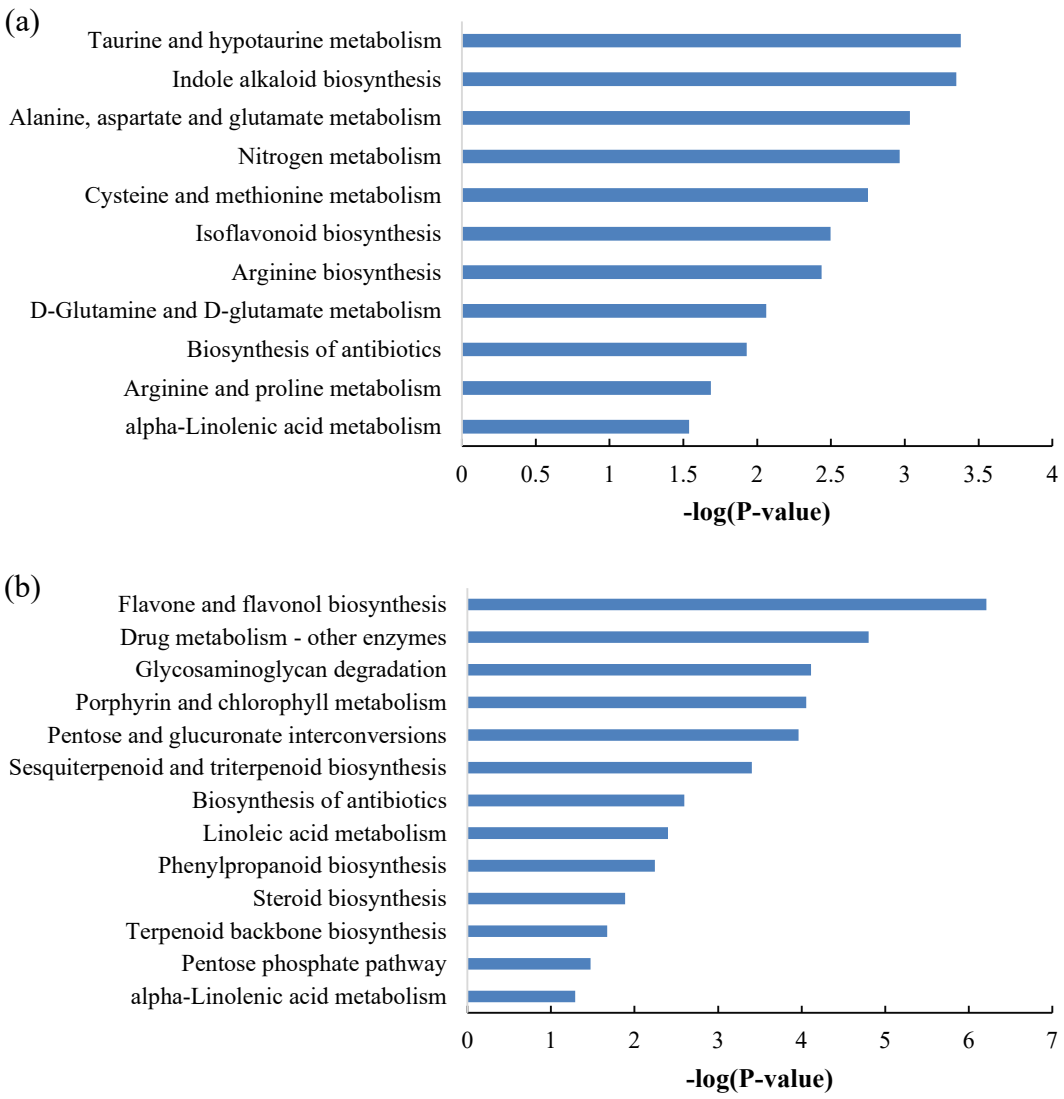


Figure 5. Pathways enriched in upregulated genes (a) and downregulated genes (b) of comparison between RRIM HYDROBEST™ and TPD.

CONCLUSIONS

In conclusion, TPD mechanism was not specific to any pathway but it can be associated with the rubber tree response to oxidative stress as most of TPD-related genes have some roles in increasing reactive oxygen species in the cell. The use of RRIM HYDROBEST™ had the effect of increasing latex yield as well as decreasing the incidence of TPD which could be due to the increased level of amino acids available to the cells and reduced synthesis of different classes of isoprenoids. In this case, the natural rubber could be the main class of isoprenoid to be produced.

AUTHOR CONTRIBUTION

AZN conceived, designed and performed the analysis, wrote the paper, and checked and approved the submission.

CONFLICT OF INTEREST

The author declares there is no conflict of interests.

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