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Comparative transcriptome analysis of Nile tilapia (*Oreochromis niloticus*) under different health conditions associated with tilapia lake virus disease (TiLVD)

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Abstract Transcriptomic analysis was performed in liver tissue of 3 different treatments of Nile tilapia including normal or healthy (no *Tilapia tilapinevirus* or tilapia lake virus (TiLV) infection) fish (TiN), moribund TiLV-infected fish (TiB) and recovered fish from TiLV infection (TiR). The obtained results revealed that diverse genes were expressed among TiN, TiB and TiR that could lead to the different functions and involving pathways. Differentially expressed genes (DEGs) in TiR and TiN showed similar read numbers and expressed genes whereas the DEGs result in TiB was distinctly detected. All DEGs of these 3 samples revealed that the expression of genes involved in metabolic pathways was high and its expression levels were remarkably differences. Gene ontology (GO) enrichment analysis and Kyoto encyclopedia of genes and genomes (KEGG) was analyzed comparatively among 3 pairs of tilapia samples; (1) TiB vs. TiN (2) TiR vs. TiN and (3) TiR vs. TiB. The results showed that genes encoding proteins involving in apoptosis, peroxisome and phagosome were detectable and significantly different in TiB vs. TiN and TiR vs. TiB but absent in TiR vs. TiN. However, genes involved in oxidative phosphorylation were only detected in TiR vs. TiN. A set of genes involved in proteasome, a sophisticated protease complexes that function in regulated degradation of unneeded or damaged proteins by proteolysis, was only appeared in TiB vs. TiN. These results are important knowledge regarding a new emerging disease leading to surveillance, cultural and farm management practices and bioactive compound development to reduce losses affected by TiLV.

Keywords: Nile tilapia, Tilapia lake virus (TiLV), *Tilapia tilapinevirus*, Transcriptome analysis

Introduction

Nile tilapia (*Oreochromis niloticus*) is one of the most important Thailand's aquaculture species. It is currently accounts for the more than 55% of

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production and estimated value around 37% of the country's total freshwater organism production (DOF, 2021). Tilapia is considered as a suitable candidate in various aquaculture strategies because of its benefits including, rapid growth, uncomplicated dietary requirement and tolerance against a wide range of environmental conditions. (Soto *et al*, 2019). However, tipalia culture has been confronted with disease challenges caused by multitude pathogens, bacteria, parasites, and viruses.

Among the viral diseases in tilapia, tilapia lake virus disease (TiLVD), that is due to *Tilapia tilapinevirus* or tilapia lake virus (TiLV), has seriously threatend the global tilapia aquaculture in recent years (Jansen *et al*., 2019). TiLVD has been classified as an emerging disease by the World Organisation for Animal Health (OIE) and is currently being evaluated for inclusion on the list of the reportable finfish disease list (OIE, 2020). TiLV, a single-stranded, negative-sense RNA virus with an envelope, belongs to the Orthomyxoviridae family (ABRS, 2020). There has been reported that TiLV infection in fish manifests through various including lethargy, loss of appetite, abnormal behaviours like surfacing and ceasing schooling). In addition, anaemia, pallor, body discoloration, exophthalmia, abdominal swelling, skin congestion, and erosion may be observed (Dong *et al*., 2017; Surachetpong *et al*., 2017). TiLV has noticeably been associated with summer mortality syndrome, one-month mortality syndrome and syncytial hepatitis disease which have been demonstrated by Nicholson *et al*. (2017), Surachetpong *et al*. (2017) and Del-Pozo *et al*. (2017), respectively. In Thialand, massive mortality of tilapia especially that of red tilapia fingerlings during the first month after being subjected to floating cages has been recorded (Dong *et al*., 2017). Experimental injection of TiLV through cohabitation and intraperitoneal challenge can cause TiLVD, in addition, high mortality is detected within 2 weeks (Eyngor *et al*., 2014; Surachetpong *et al*., 2017).

The advent of genome sequencing via next generation sequencing (NGS) since 2005, particularly its application in transcriptomic analysis, has empowered researchers to address numerous challenges in aquaculture (Liu *et al*., 2021). Transcriptomics through RNA sequencing (RNA-seq) discloses the gene expression patterns across the genome and plays a role in understanding the molecular mechanisms underlying immunity responses to various pathogens. This technique provides the identification and quantification data of differentially expressed genes that could be beneficial for application in aquaculture. For example, the study on Nile tilapia's immune response to *Streptococcus agalactiae* has shown many differentially expressed immune-related genes within the transcriptome including several significant genes associated with pathogen attachment (Zhu *et al*., 2017). Besides, gene onthology,

obtained data from transcriptome analysis, is also used to systematic characterization of the functions of gene and gene product across species. This facilitates computational predictions of unknown gene functions (Zhao *et al*., 2020).

This study aimed to analyze differences in mRNA transcription levels between tilapia infected with TiLV, recovered tilapia, and healthy one.

Materials and methods

Fish samples and TiLV infection

Healthy Nile tilapia (approximately 30 g) was acclimatized before being randomly detected for the TiLV infection through PCR following the method described by Eyngor *et al*. (2014) and Tsofack *et al*. (2016). Thirty fish were transferred to a 200-L tank; 3 fish were collected as the healthy one (TiN) while the rest were intraperitoneally injected with 100 µL supernatant from TiLV-infected fish tissue. Severe fish with the cinical signs of TiLVD were separated and collected as moriboud sample (TiB). The TiLV-injected fish that showed clinical signs then became exhibiting normal behavior was collected as a recovered tilapia (TiR). Liver tissue was isolated from the above 3 different health conditions of tilapia and kept in RNAlater Stabilization Solution (Thermo Fisher Scientific). All tissue samples were then frozen in liquid nitrogren and stored in -80^{0}C for further downstream analysis.

RNA extraction

Total RNA was extracted from the liver samples using the RNeasy Mini kit (Qiagen), followed the manufacturer's procedure. The contaminated genomic DNA was eliminated from the extracted RNA by RNase-free DNase I (Thermo Fisher Scientific) treatment. Before library construction, RNA quality and integrity was analyzed using Nanadrop, agarose gel electrophoresis and Agilent 2100 for RNA purity, RNA degradation and potential contamination and RNA integrity, respectively.

Library construction and RNA-Seq

One µg of the obtained RNA was used for library construction. The transcriptome library was prepared using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB) following manufacturer's protocol. Subsequently, each sample was added unique index codes for sequence attribution. Briefly, mRNA

was isolated from the DNase I-treated RNA using poly-T oligo-attached magnetic beads. The enriched mRNA was fragmented randomly and reversetranscribed with M-MuLV Reverse Transcriptase (RNase H^-) and random hexamer primer. After first-strand synthesis, a second-strand cDNA was generated by nick-translation. Following second-strand cDNA synthesis with DNA Polymerase I and RNase H, the samples underwent purification and ligation with Illumina sequencing adapters and index. Size selection for the cDNA, preferentially ~150-200 bp, was employed and then enriched for the final sequencing library generation. The constructed libraly quality was quantified on the Agilent Bioanalyzer 2100 system. These libraries were subsequently sequenced through the Illumina platform. The paired end reads of 125 bp/150 bp in length were generated. Analysis of differentially expressed genes (DEGs) of two conditions/groups; (1) TiB vs. TiN (2) TiR vs. TiN and (3) TiR vs. TiB, was performed using the DESeq R package (1.18.0). To control of false discovery rate, the P-values were adjusted using the Benjamini and Hochberg's approach. DESeq analysis designated genes with an adjusted P-value less than 0.05 (P-value < 0.05) as differentially expressed.

RNA-Seq data processing and analysis of differential gene expression

Prior to downstream analyses, raw sequencing data or raw read (fastq format) were processed to remove reads containing adapter, reads containing ploy-N and low-quality reads from raw data. This process ensures high-quality clean reads for subsequent analyses. The reference genomes and gene model annotations were directly downloaded from a genome database. Bowtie v2.2.3 was used to build an index of the reference genome allowing for paired-end clean reads alighment to the Nile tilapia reference genome (project PRJNA344471) using TopHat v2.0.12. Finally, HTSeq v0.6.1 was used to quantify the number of reads mapped to each gene.

Gene Ontology (GO) analysis

The GOseq R package was used to implement GO enrichment analysis of DEGs. GO terms with a corrected P-value < 0.05 were considered significantly enriched by DEGs. Additionally, KOBAS software was utilized for Kyoto encyclopedia of genes and genomes (KEGG) analysis, identifying the statistically enriched DEGs in KEGG pathways.

Results

Transcriptome sequencing

A total of 45,202,472, 44,330,984 and 48,340,872 raw reads were obtained for TiN, TiB and TiR, respectively. After removing adaptor and filtering, the clean reads of TiN, TiB and TiR were 43,551,272, 42,896,260 and 46,820678, respectively that showed 0.02% error rate for each sample. The average Q30 percentage and GC content of all samples were more than 91% and 48%, respectively, indicating the obtained transcriptome data were reliable. Upon mapping, approximately 71-73% clean reads were compared to the tilapia reference genome while 71.51%, 69.50% and 71.15% showed uniquely mapped for TiN, TiB and TiR, respectively (Table 1).

Table 1. Sequencing and mapping statistics of the liver transcriptomes of Nile tilapia

Sample	Raw reads	Clean reads	Q20 (%)	Q30 (%)	GC count (%)	Total mapped	Uniquely mapped
TiN	45202472	43551272	96.64	91.75	49.86	30699900 (71.57%)	29814642 (69.5%)
TiB	44330984	42896260	96.59	91.72	48.90	30699900 (71.57%)	29814642 (69.5%)
TiR	48340872	46820678	96.56	91.66	49.22	34518998 (73.73%)	33313242 (71.15%)

Differentially expressed genes (DEGs)

The expression levels of DEGs in the 3 groups with different health conditions (TiN, TiB and TiR) were evaluated. The number of uniquely expressed genes within each sample and those of the genes expressed in 2 or 3 samples present with the overlapping regions were shown via the coExpression_venn Diagram. The diagram revealed 9,319 genes expressed in all 3 conditions while 214, 786 and 342 genes were uniquely expressed in TiN, TiB and TiR, respectively (Figure 1).

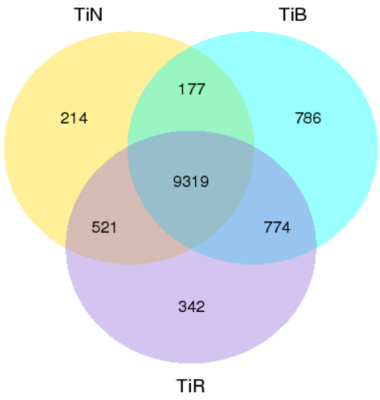

Figure 1. coExpression_venn diagram of differentially expressed genes (DEGs) in liver of normal (TiN), moriboud TiLV-infected (TiB) and recovered tilapia (TiR)

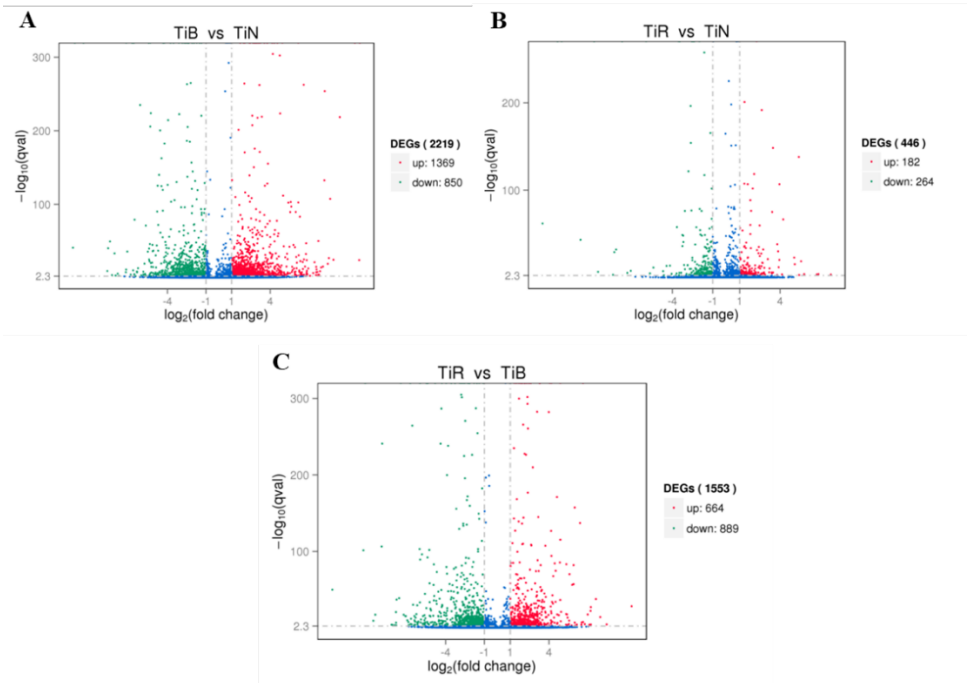

Figure 2. Analysis of differentially expressed genes (DEGs) via volcano plot, identified between normal (TiN) and moriboud tilapia (TiB) **(A)**, normal and recovered tilapia (TiR) **(B)** and moriboud and recovered tilapia **(C)**. Up- and downregulated DEGs are shown in red and green, respectively. Blue dots denote genes that are not significantly changed

To explore the differential gene expression among the different health conditions associated with TiLV infection, the absolute value of log2 fold change in expression > 1 and q-value < 0.005 were set as a threshold level to retrieve the DEGs. Overall, 2,219, 446 and 1,553 DEGs were detected on comparing TiB vs. TiN (1,369 up- and 850 down-regulated genes), TiR vs. TiN (182 up- and 264 down-regulated genes) and TiR vs. TiB (664 up- and 889 down-regulated genes), respectively (Figure 2). In GO distribution, the highest enriched subcategory within the biological process was oxidation-reduction process GO:0055114 while oxidoreductase activity GO:0016491 was the largest enriched GO term with molecular function. Moreover, cluster analysis to cluster genes with similar patterns were also performed as shown in Figure 3. Among these 3 conditions, many gene clusters in TiN were distinctly expressed.

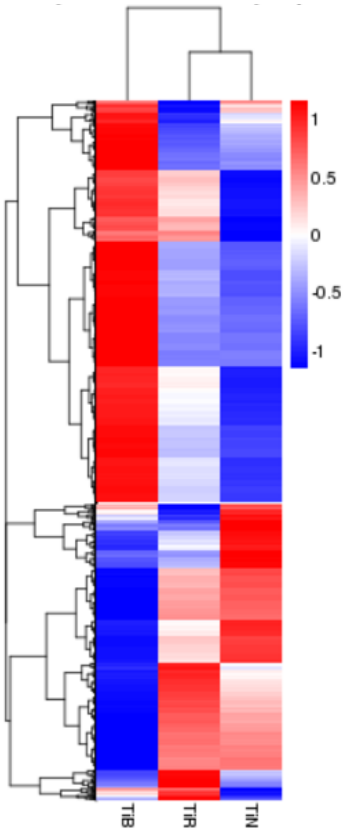

Figure 3. Cluster analysis of DEGs. DEGs were clustered based on their $\log_{10}(\text{FPKM}+1)$ value. Red and blue denotes genes with high expression levels and low expression levels, respectively. The color ranges from red to blue reflects the $\log_{10}(\text{FPKM}+1)$ magnitude (large to small)

Gene ontology (GO) and Kyoto encyclopedia of genes and genomes enrichment (KEGG) of differentially expressed genes (DEGs)

To explore the functional roles of the identified DEGs, the GO enrichment analysis was carried out. The obtained result revealed enrichment in 2 GO categories including biological process and molecular function. Notably, no significant enrichment was observed for cellular component terms. On comparing TiN vs. TiB, the most significant enriched GO terms in biological process category were biological process, metabolic process and single-organism metabolic process while within the molecular function category were catalytic activity and oxidoreductase activity (Figure 4A). Comparative analysis of TiB and TiN (Figure 4B) revealed that the most significant enriched GO terms in biological process category were metabolic process followed by single-organism metabolic process and oxidation-reduction process. Similarly, enriched GO terms in molecular function category were catalytic activity and oxidoreductase activity suggesting potential alterations in cellular metabolism. Likewise, the comparison between TiR and TiB (Figure 4C) showed enrichment in similar biological process catagories, the most enriched subcategories were biological process, metabolic process and single-organism metabolic process. The molecular function category was also significantly enriched in catalytic activity and oxidoreductase activity.

KEGG pathway analysis was performed to analyze the involvement of DEGs in various signaling pathways. This analysis revealed enrichment of metabolic pathways in all 3 comparisons: TiB vs. TiN, TiR vs. TiN and TiR vs. TiB. Interestingly, the genes in phagosome, lysosome, proteosome, and apoptosis were not noticeably enriched in TiR vs. TiN while they were enriched in TiB vs. TiN and TiR vs. TiB. The genes in oxidative phosphorylation and peroxisome were significantly enriched in only TiR vs. TiN and TiB vs. TiN, respectively (Figure 5).

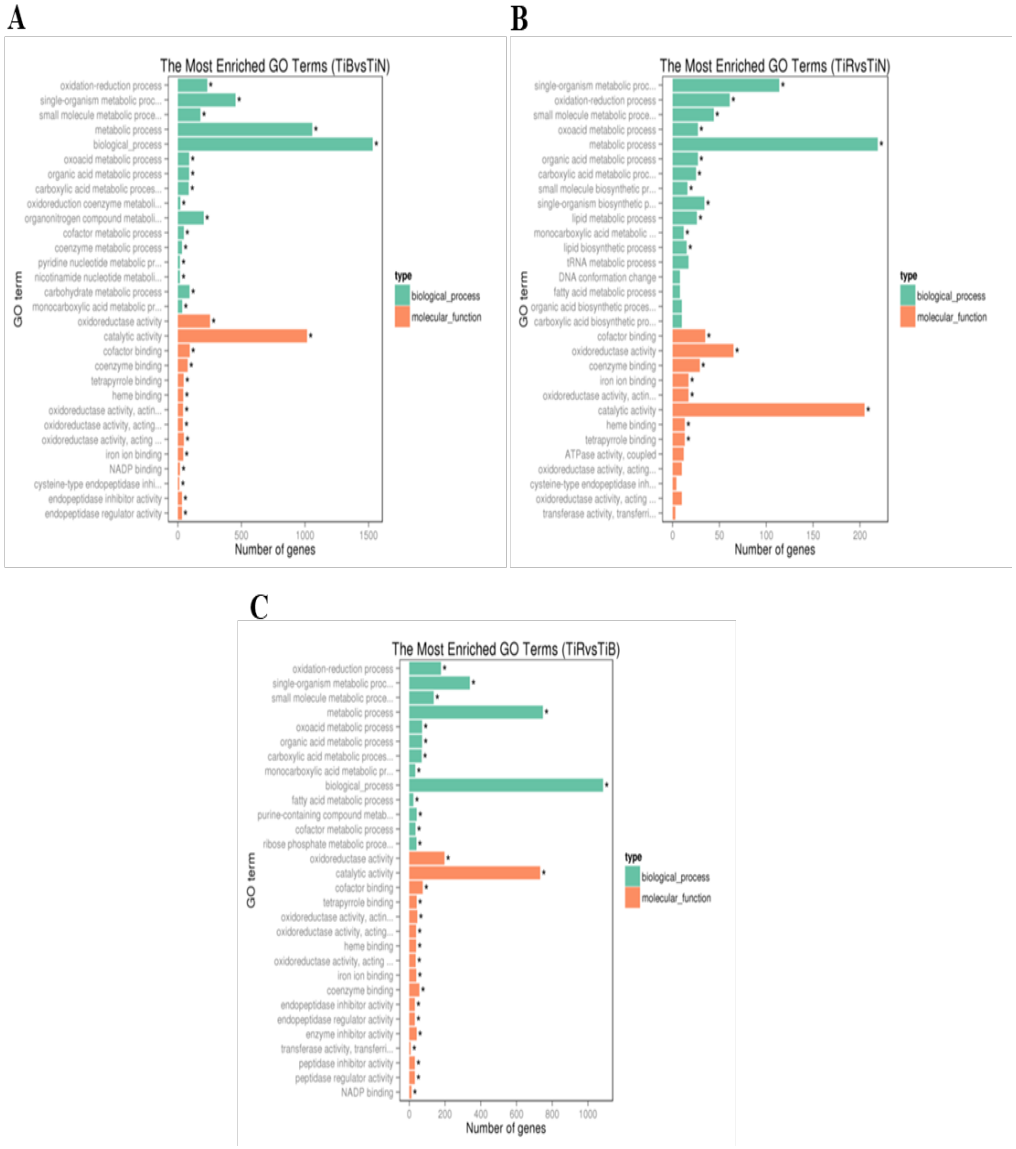

Figure 4. Gene ontology (GO) classification analyses of DEGs in the liver of Nie tilapia compared between normal (TiN) and moriboud tilapia (TiB) **(A)**, normal and recovered tilapia (TiR) **(B)** and moriboud and recovered tilapia **(C)**; Bars represent the significance level of the enriched GO terms

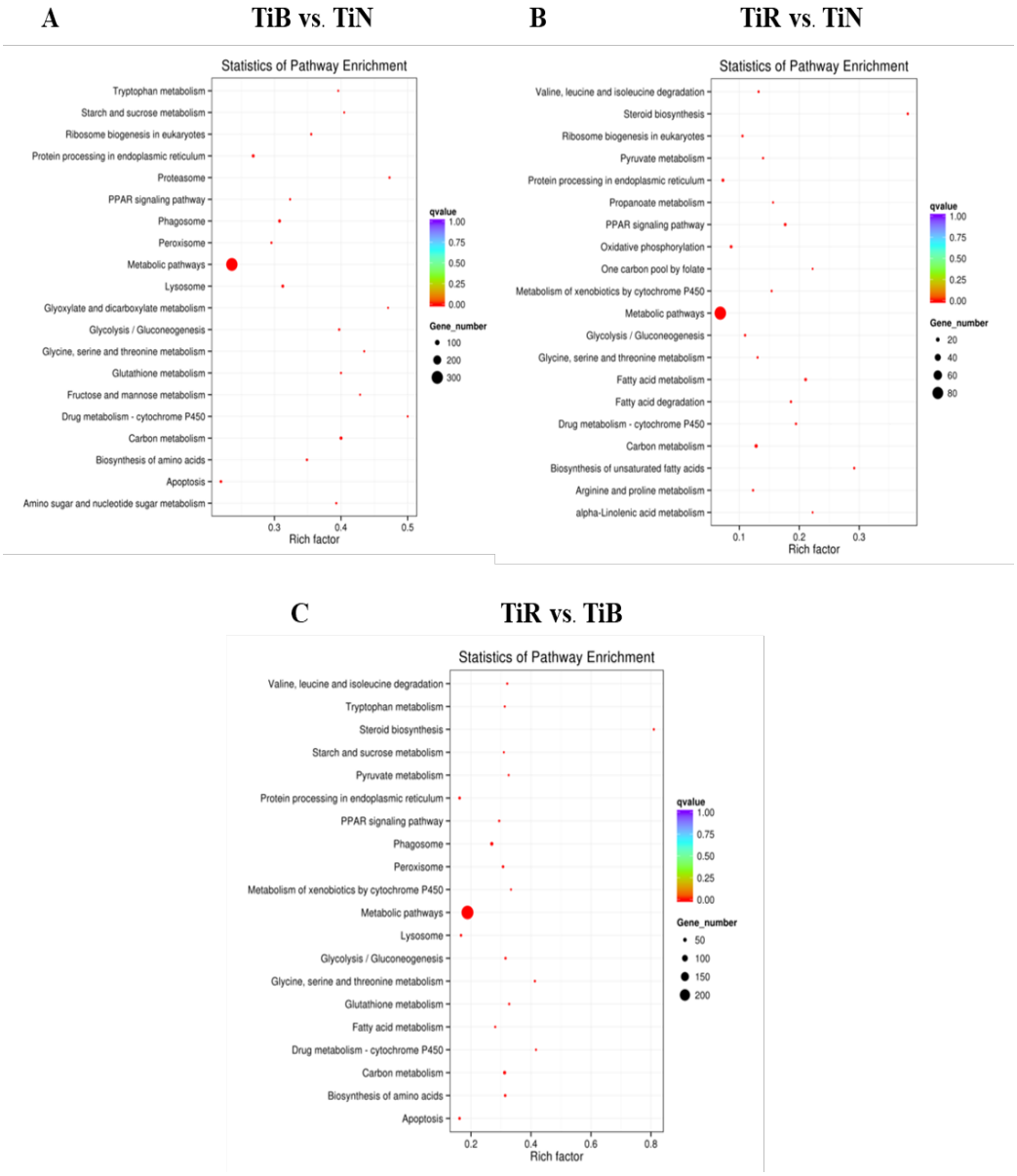

Figure 5. Significant KEGG pathway classifications of DEGs compared between normal (TiN) and moriboud tilapia (TiB) **(A)**, normal and recovered tilapia (TiR) **(B)** and moriboud and recovered tilapia **(C)**; Bars represent the significance level of the enriched GO terms

Discussion

TiLV has recently been reported for its spread and negative effects on tilapia culture and industry worldwide including Thailand (Eyngor *et al.*, 2014; Dong *et al.*, 2017; Surachetpong *et al.*, 2017). Many researchers have focused on diverse aspects relevant to TiLV and TiLVD including its etiology (Tattiyapong *et al.*, 2017; Acharya *et al.*, 2019), host range and life stages for infection (Eyngor *et al.*, 2014; Dong *et al.*, 2017; Abdullah *et al.*, 2018; Jaemwimol *et al*., 2018; Aich *et al*., 2022), mode of transmission (Eyngor *et al*., 2014; Dong *et al*., 2017), pathology and pathogenesis (Eyngor *et al*., 2014), and developed diagnosis to increase the sensitivity (Eyngor *et al.*, 2014; Surachetpong *et al.*, 2017; Tsofack *et al.*, 2016; Waiyamitra *et al.*, 2018). Moreover, the responses as well as the procedure to increase the resistance of tilapia host against this virus have also been highlighted (Eyngor *et al.*, 2014; Tattiyapong *et al*., 2020; Sood *et al*., 2021).

In this study, we aimed to get insight into the molecular mechanism of Nile tilapia related to the TiLV infection; the severe or moriboud situation and those when the fish recovered. Transcriptome analysis in liver tissue of Nile tilapia exhibiting different 3 health conditions including normal, TiLV infected- and moribund fish were conducted through RNA- Seq followed by comparative analysis. RNA-Seq has currently employed for studying the hosts' transcriptional responses to several pathogens (Kumar *et al*., 2017; Sood *et al*., 2021; Verma *et al.*, 2021). The obtained DEGs revealed that many gene clusters differently expressed. The data of GO enrichment indicated that oxidoreuctase activity and oxidation- reduction process were the largest significantly enriched within the category of molecular function and biological process. KEGG enrichment analyses further suggested the identification of differentially enriched pathways, for example, glycolysis/ gluconeogenesis, phagocytosis, lysosome, apoptosis, and oxidative phosphorylation.

Oxidoreductase, a large group of enzymes including oxidase, oxygenase, dehydrogenase, peroxidase, etc., plays an important role in redox reaction in organisms. This enzyme plays a multifacted role in cellular metabolism. It participates in diverse pathways, including biomolecule synthesis, degradation of certain molecules, removal of unwanted compounds, and metabolism of exogenous molecules (Braune *et al.*, 2019). Notably, oxidoreductase enzyme catalyzes reactions in glycolysis, TCA cycle, electron transport chain, and oxidative phosphorylation. Oxidoreductase enzymes functions in either oxidative stress or reactive oxygen species (ROS) scavenging system or antioxidant system. In addition, there has been reported that this group of

enzymes may be associated with phagocytosis, tissue and cell apoptosis and other immune responses (Kumari *et al.*, 2014; Biller and Takahashi, 2018; Johnstone and Chaves-Pozo, 2022).

Similarly, Sood *et al*. (2021) have previously demonstated the different transcriptome analysis compared between the mormal and TiLV-infected tilapia and have reported that the genes involving in immune responses. The immune-realated responses including antigen processing and presentation, MAPK signaling, JAK-STAT pathways, necroptosis, apoptosis, chemokine signaling, NF-kB, interferon, and acute phase response are differentially expressed. Apoptosis is a physiological cell death defined by specidic phenomena including DNA fragmentation, chromatin condensation, cell shrinkage, formation of apoptotic bodies (Monteiro *et al*., 2009). There has been supposed that apoptosis of the infected cell could serve as a potential mechanism to restrict the spread of pathogens including virus (Barber, 2001). Therefore, some of vital genes in apoptosis are upregulated to support the host defense. Conversely, some genes are downregulated in TiLV-infected tilapia since TiLV may be albe to counteract the host defense mechanisms to successfully infection Sood *et al*. (2021). Our result of KEGG enrichment showed that phagocytosis was enriched in comparison between TiB vs. TiN and TiR vs. TiB suggesting that phagocytosis could play a significant in TiLV infection. This data is comparable to the report of Nainu *et al*. (2017) suggesting that apoptosis-dependent phagocytosis of virus-infected cells acts as a mechanism of the innate immune respone to eliminate invading viruses. Besides, lysosome was observed to be enriched. Lysosome is a membrane-bound organelle in eukaryotic cells that involves in endocytosis, autophagy and phagocytosis. There are diverse hydrolytic enzymes such as proteases, lipases, nucleases, etc. contained in lysosome that could serve to digest complex into building block subsequently used for anabolism (Sachdeva and Sundaramurthy, 2020). Lysosomal acid hydrolases are thought to be employed to neuter pathogens, however, some pathogens adap for their survival by avoiding trafficking to lysosomes (Tang *et al.*, 2015: Martinez *et al.*, 2018). Moreover, a set of genes involved in proteasome, a sophisticated protease complexes that function in regulated degradation of unneeded or damaged proteins by proteolysis, was only appeared in TiB vs. TiN. Based on the DEGs and KEGG, we found that not only the genes and pathways involving in immunity but also the oxidative phosphorylation was enriched particularly in the comparison between TiR vs TiN. This may be due to energy is much required for recovery process and oxidative phosphorylation is the final step in cellular

respiration mainly involved in ATP synthesis via coupled movement of eletron through electron transport chain.

In conclusion, transcriptomic analysis conducted in this present study suggesting that the different states of TiLV infection led to the dissimilar expressed genes. TiLV was able to significantly induce oxidative stress and cell damage leading to apoptosis in the moriboud TiLV-infected tilapia compared the normal and recovered fish. In addition, energy metabolism may involve in the recovery procress of the host. This study provides the data underlying molecular mechanisms of TILV infection and the recovery process. These results deliver knowledge regarding host response to TiLV that can be applied for surveillance, cultural and farm management practices and bioactive compound development to reduce losses affected by TiLV. However, further studies are needed for understand pertinent mechanisms to develop farm management practices and bioactive compound to enhace the survival of tilapia and reduce losses affected by TiLV.

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