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Transcriptome of white shrimp *Litopenaeus vannamei* induced with rapamycin reveals the role of autophagy in shrimp immunity

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ABSTRACT

Autophagy plays a vital role in innate and adaptive immunity against invading microorganisms, such as virus and bacteria. However, the mechanism underlying autophagy in shrimp is still limited. In our study, we challenged white shrimp *L. vannamei* with rapamycin to induce autophagy and employed Solexa/Illumina high-throughput RNA-seq method to examine the differences of transcriptome from gills of shrimps treated with or without rapamycin. More than 22.64 Gb raw data were produced, which were assembled into 62, 503 unigenes, with 14,126 unigenes over 1 kb in length. We then performed differential expression analysis and identified a total of 3050 differentially expressed genes (DEGs). Among them, 1456 were upregulated and 1594 were downregulated. We further annotated DEGs by matching against non-redundant protein sequence (Nr), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups of proteins (COG), euKaryotic Orthologous Groups (KOG), Gene ontology (GO), and Pfam databases. The assembled and annotated DEGs will facilitate our understanding of the molecular mechanism underlying autophagy and promote the studies on the role of autophagy in innate immunity of *L. vannamei* and other crustaceans.

1. Introduction

Autophagy is a highly conserved homeostatic process which involves the sequestration of material of endogenous or exogenous origin to autophagosomes and subsequently delivery to lysosomes for degradation [1]. Autophagy takes place at a basal level in virtually all eukaryotic cells, from yeast to humans, and plays a housekeeping function to maintain cytohomeostasis by removing misfolded proteins and damaged organelles [2]. Autophagy can be activated in response to various pathological and physiological states, such as oxidative stress, immune cell activation endoplasmic reticulum (ER) stress and cell starvation [3]. Autophagy primarily functions as a protective mechanism during nutrient deprivation by degrading carbohydrates, lipids and proteins to maintain energy homeostasis [4]. Autophagy also plays a significant role in organism development and cell differentiation [5]. Recently, autophagy has been reported to play a crucial function in innate and adaptive immunity against invading microorganisms, and impairment of autophagy will enhance susceptibility to infection [6].

White shrimp *Litopenaeus vannamei* is the major aquaculture crustacean species in the world [7]. Shrimp aquaculture industry have been threatened by the outbreak of different viruses, such as white spot syndrome virus (WSSV) and taura syndrome virus (TSV) [8,9]. Transcriptome analysis is a powerful tool to determine transcript abundance and transcriptomic profiles with a broad dynamic range and has been employed to identify gene involved in virus infection in shrimps, however, information on the molecular mechanisms of autophagy in shrimp is still limited. Rapamycin, an inhibitor of mTOR signaling pathway, is generally used as an inducer of autophagy [10]. In the present study, we analyzed the transcriptome of gills from shrimps treated with rapamycin using Solexa/Illumina high-throughput se-

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Table 1
Summary of clean reads statistics by Illumina sequencing.

Samples	Read Number	Base Number	GC Content	%≥Q30
control	45,597,715	11,490,624,180	49.32%	91.16%
rapamycin	44,249,643	11,150,910,036	48.70%	91.39%

Table 2
Summary of *de novo* assembly results of the transcriptome.

Length Range	Transcript	Unigene
300–500	40,960(39.74%)	30,540(48.86%)
500–1000	30,261(29.36%)	17,837(28.54%)
1000–2000	19,020(18.45%)	9406(15.05%)
2000+	12,824(12.44%)	4720(7.55%)
Total Number	103,065	62,503
Total Length	108,928,838	52,245,715
N50 Length	1600	1136
Mean Length	1056.89	835.89

quencing approach, generating of 22.64 Gb clean data, which were assembled into 62, 503 unigenes, with 14,126 unigenes over 1 kb in length. Annotation of all unigenes by matching against non-redundant protein sequence (Nr), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups of proteins (COG), euKaryotic Orthologous Groups (KOG), Gene ontology (GO), and Pfam databases obtained 28,169 annotated unigenes. We then analyzed the gene expression profiles in the control and rapamycin-treated group and identified 3050 differentially expressed genes (DEGs), including 1456 upregulated and 1594 downregulated. We further annotated DEGs by matching against GO, KEGG, and COG databases and identified 1738 annotated unigenes.

2. Materials and methods

2.1. Shrimp culture and autophagy induction

Shrimp *Litopenaeus vannamei* (approximately 10–15 g body weight) culture was performed as previously described [11]. In brief, shrimp

Table 3
Summary of the annotations of all unigenes.

Database	Annotated Number	percentage (%)
Annotated in Nr database	25284	89.76
Annotated in Swiss-Prot database	16221	57.58
Annotated in GO database	12687	45.04
Annotated in COG database	11781 41.82	
Annotated in KOG database	20350	72.24
Annotated in KEGG database	15325	54.40
Annotated in Pfam database	22234	78.93
All Annotated unigenes	28169	100

was cultured in 80 L aquariums filled with air-pumped circulating seawater at 25 °C. They were fed with commercial diet before and during experiments. Autophagy was induced by treating shrimp with rapamycin which is widely used to induce autophagy by inhibiting target of rapamycin receptor (TOR) kinase. 50 μmol/shrimp rapamycin (Cell Signaling Technology, USA) was intramuscularly injected into shrimp by using a syringe with a 29-gauge needle. The shrimp gills (3 gill tissues were pooled in control and rapamycin-treated group, respectively) were collected after injection for 24 h for total RNA extraction.

2.2. Total RNA isolation

For each group, three shrimps were randomly chosen and their gills were used for total RNA isolation. Total RNA was extracted with TRIzol reagent (Invitrogen, USA) by following the manufacture's instruction and treated with DNase I (Takara, Japan) according to the manufacturer's manual to remove any genomic DNA. The concentration of total RNAs was estimated by spectrophotometry at OD_{260} (Eppendorf, Germany) and their integrity were examined by electrophoresis on a 2% agarose gel. The extracted RNAs were stored at −80 °C for later use.

2.3. Library construction and deep sequencing

Library construction and sequencing was carried out by Biomarker Technologies, INC. Briefly, an amount of 3 μg total RNA from each group was used as input material for mRNA preparation and cDNA

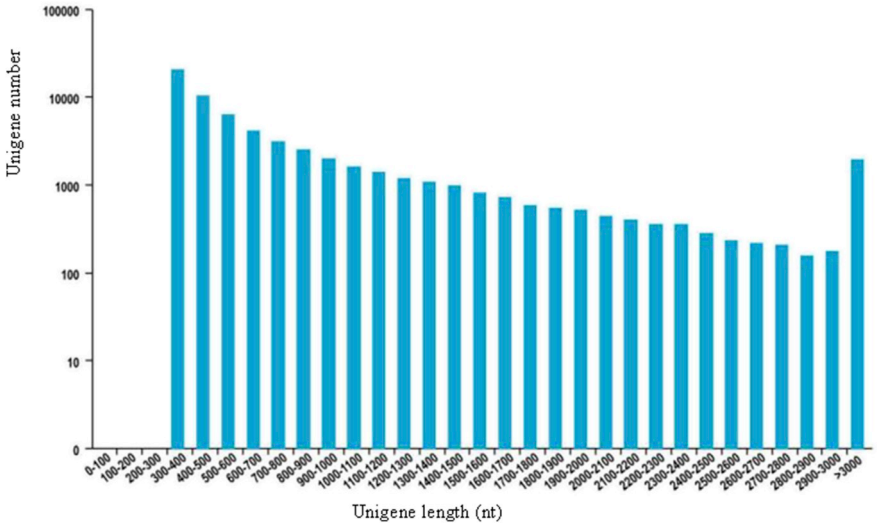

Fig. 1. The length distribution of unigenes.

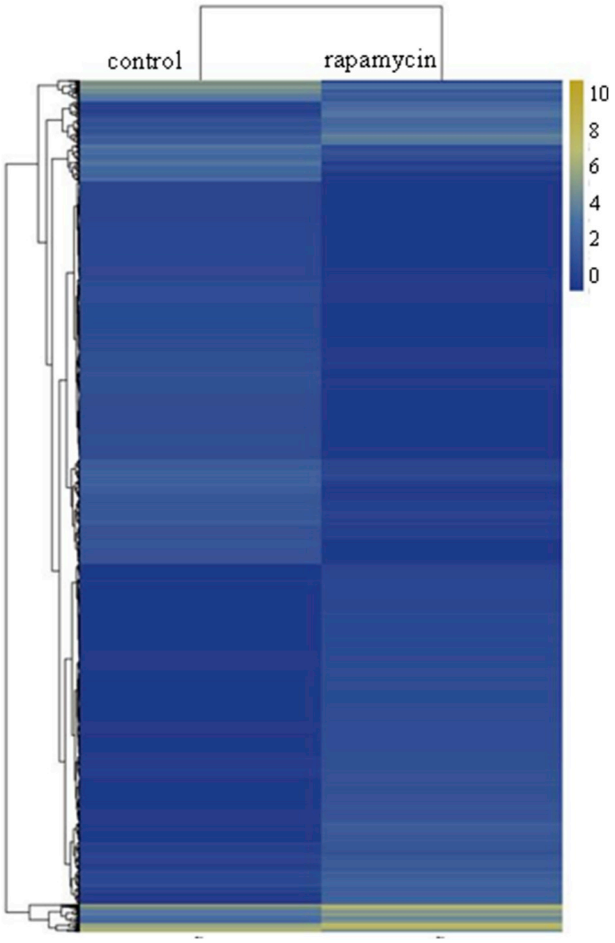

Fig. 2. Heat map of differentially expressed genes in control and rapamycin challenged transcriptome of *Litopenaeus vannamei*.

synthesis. Sequencing libraries were constructed using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) according to manufacturer's instructions and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq 2500 platform (Beijing Biomarker Technologies Co., Ltd. Beijing, China) with one lane of 2 × 150 base pairs (bp) reads from both ends of the fragments (paired ends).

2.4. Transcriptome assembly and gene annotation

The invalid reads obtained from sequencing machines including adapters, unknown or low-quality reads were removed to avoid negative effect on subsequent bioinformation analysis. The 150-bp high-quality clean reads were used for the downstream analysis. *De novo* transcriptome assembly was performed with Trinity software, a short-reads assembling program as previously reported by using the combined sequence data from both the Rapamycin-challenged and the control samples [12]. The clean reads were firstly randomly clipped into overlapping K-mers and connected into longer fragments named contigs by comparing the overlaps in different clean reads. These contigs were clustered into unigenes by pair-end joining. All clean reads were compared back with contigs. The clean reads that can be mapped in unigenes were called mapped reads which were employed in subsequent analysis. Unigenes from the *De novo* assembly were further processed for sequence annotation by using BLASTX in Nr, Swiss-Prot, KEGG, COG, KOG, GO and HMMER in Pfam databases.

2.5. DEGs identification and annotation

It has been reported that digital gene expression profiling by transcriptome analysis (high-throughput RNA sequencing) is highly reproducible, so it might be unnecessary to have biological replicates for

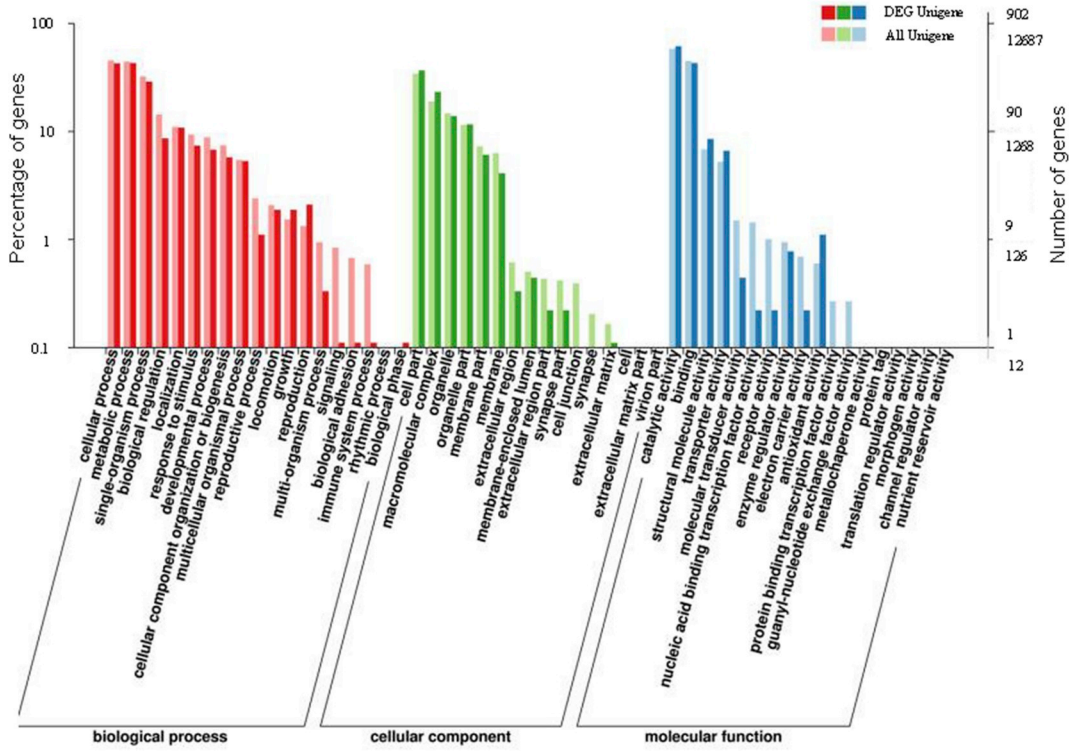

Fig. 3. GO terms and annotation of the integrated transcriptome assembly.

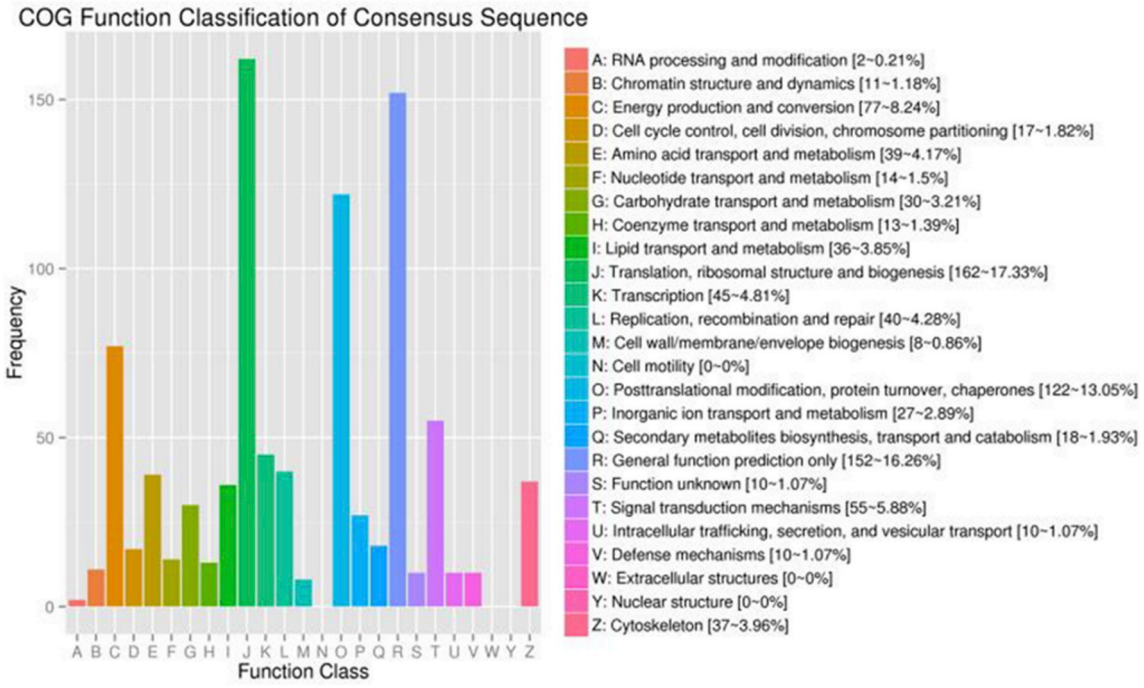

Fig. 4. COG function classification of different expression genes.

digital gene expression profiling by high-throughput RNA sequencing [13,14]. The relative transcript abundance was expressed using RPKM (Reads Per Kb per Million reads) as previously reported [15]. EBSeq approach was employed to examine DEGs between the control group and the rapamycin-exposed group [16]. The results of statistical tests were corrected by multiple test with the Benjamini–Hochberg false discovery rate control (FDR < 0.01). The expression of transcripts was considered to be significantly different if the adjusted p value was less than 0.01 ($p < 0.01$) and a fold change greater than 2 ($FC \geq 2$) in expression across libraries was presented.

Table 4
COG category "Posttranslational modification, protein turnover, chaperones".

gene	regulated
heat shock protein 21 kDa	Up
heat shock protein 70 kDa	Up
heat shock protein 90 kDa	Down
ubiquitin-conjugating enzyme	Down
ubiquitin-conjugating enzyme	Down
ubiquitin-activating enzyme E1	Down
ubiquitin carboxyl-terminal hydrolase	Down
proteasome alpha subunit	Up
proteasome beta subunit	Up
26S proteasome subunitP45	Down
proteasome/cyclosome repeat family protein	Down
26S proteasome regulatory complex, subunit RPN2	Down
26S protease regulatory subunit 6B	Down
AAA family ATPase	Down
DnaJ domain containing protein	Down
DnaK protein	Up
peptidyl-prolyl cis-trans isomerase	Up
plasminogen-like isoform X1	Down
serine protease	Down
TCP-1/cpn60 chaperonin family protein	Up
telomerase-associated protein p20	Down
thermophila SB210	Up
thioredoxin-dependent peroxide reductase	Up
transitional endoplasmic reticulum ATPase	Up
UBA/TS-N domain containing protein	Down
vacuolar sorting protein	Down

3. Results

3.1. Transcriptome sequencing and assembly

After removing the adapter sequences and low-quality sequences, a total of 22.64 Gb clean data (Q30 ≥ 91.16%) were produced by using Illumina HiSeq 2500 containing 89,847,358 clean reads with 45,597,715 reads from control group and 44,249,643 reads from rapamycin-treated group. The GC content of clean reads is 49.32% in the control group and 48.70% in rapamycin-challenged group, respectively (Table 1). The combined clean reads from the two libraries were employed in *de novo* assembly using Trinity software and up to 103,065 transcripts with average length 1056.89 base (N50 = 1600) were

Table 5
COG category "Lipid transport and metabolism".

gene	regulated
acetyl-CoA acyltransferases	Up
acyl-CoA dehydrogenase	Up
acyl-CoA oxidase	Down
enoyl-CoA hydratase	Down
inositol-3-phosphate synthase	Up
propionyl-c arboxylase beta chain	Down

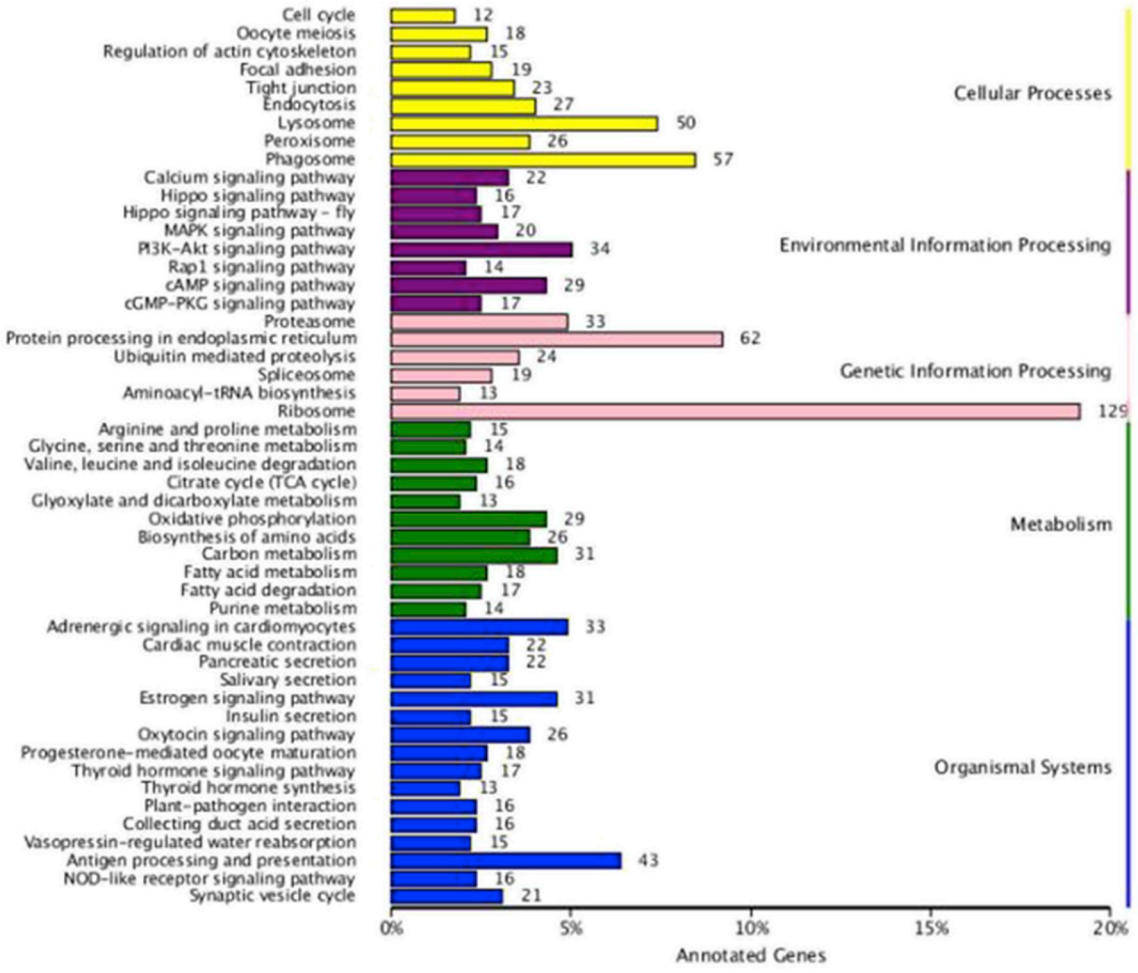

Fig. 5. Highly expressed biological pathways represented in *Litopenaeus vannamei* transcriptome retrieved from the KEGG database.

generated (Table 2). These transcripts were subsequently assembled into 62,503 unigenes with an average length of 835.89 bp and N50 of 1136. The total length of the unigenes is 52,245,715 bp, which covered 47.96% of the length of transcripts. The length distribution of unigenes was demonstrated in Fig. 1.

3.2. Functional annotation of all unigenes derived from the control and rapamycin-treated group

After ruling out the low-quality and short-length sequences, the remaining 62,503 unigenes with a minimum length of 300 bp were subjected to annotation analysis by matching unigenes against the public databases using BLASTX (E-value ≤1e-5) and HMMER programs (E-value ≤1e-10), and the annotation results are summarized in Table 3. By this method, 28,169 unigenes (45.07% of all unigenes) returned an above cut-off BLAST result. Of them, 25,284 unigenes were matched by NR (89.76%), 16,221 matched by Swiss-Prot (57.58%), 12,687 matched by GO (45.04%), 11,781 matched by COG (41.82%), 20,350 matched by KOG (72.24%), 15,325 matched by KEGG (54.40%), and 22,243 matched by Pfam database (78.93%), respectively.

3.3. Analysis of expression profile of unigenes

To identify DEGs responsible to rapamycin treatment, we conducted comparative transcriptome analysis between control group and rapamycin-treated group by measuring transcript abundance using RPKM approach with p value was less than 0.01 ($p < 0.01$) and a fold change greater than 2 (FC ≥ 2). Up to 3050 DEGs were presented between control and rapamycin-treated group, including 1456 up-regulated genes and 1594 down-regulated genes. The DEGs were further determined using hier-archical cluster analysis which produce an intuitive way to display the clustering patterns (Fig. 2).

All DEGs were mapped to terms in GO, COG and KEGG database to evaluate their function. As a result, a total of 902 DEGs were categorized into three major functional classes according to GO categories including biological progress, cellular component, and molecular function (Fig. 3). These three main GO categories were further classified into 39 subcategories. The category of biological process contained 18 subcategories. Most of the corresponding DEGs were enriched in metabolic processes, cellular processes and biological regulation in category of biological processes. The category of cellular component contained 11 subcategories. Most of the corresponding DEGs were enriched

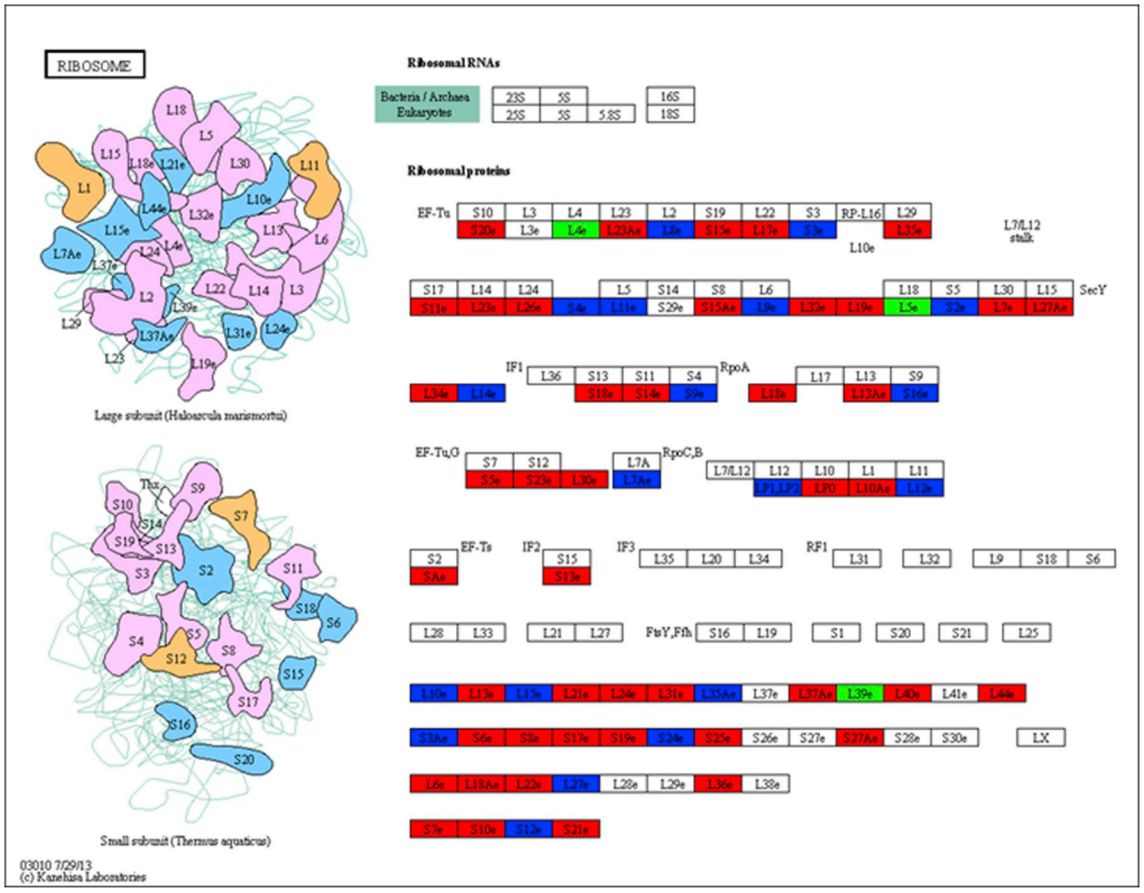

Fig. 6. KEGG related to ribosome. Components with homologues in the *Litopenaeus vannamei* transcriptome are highlighted in green (down-regulated), red (up-regualted), and blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in cell part, macromolecular complex, and organelle in category of cellular component. The category of molecular function contained 10 subcategories. Most of the corresponding DEGs were enriched in catalytic activity, binding, and structural molecular activity in category of molecular function (Fig. 3).

In the meantime, COG annotation which is important for functional annotation and evolutionary studies was conducted to predict and categorize possible roles of DEGs. A total of 731 DEGs were functionally categorized into 22 different COG categories (Fig. 4). The most enriched COG category is “translation, ribosomal structure and biogenesis”, followed by “general function predicted only” and “posttranslational modification, protein turnover, chaperones”(Table 4). It's also worth mentioning that the transcritps of some lipid transport and metabolism gene were regulated by rapamycin treated(Table 5).

KEGG is a pathway-based categorization of orthologous genes which provides useful information for gene function prediction [17]. To obtain more valuable information for functional prediction, all DEGs were mapped to KEGG database, producing 959 annotated DEGs. The 959 annotated DEGs were implicated in 50 different signaling pathways and classified into 5 different KEGG pathways, including Cellular Processes, Environmental Information Processing, Genetic Information Processing, Metabolism, and Organismal Systems (Fig. 5). The greatest number of DEGs was mapped to the pathway related to ribosome (Fig. 6) in the category of Genetic Information Processing, followed by Protein processing in endoplasmic reticulum (Fig. 7). Unexpectedly, two innate pathways, Toll-like receptor signaling pathway (Fig. 8) and NF-kB signaling pathway (Fig. 9) were founded in the KEGG.

4. Discussion

Autophagy is a conserved homeostatic process controlling energy homeostasis by balancing protein and organelle quality. Autophagy pathway and proteins play a crucial role in innate and adaptive immunity against invading microorganisms, such as virus and bacteria [18]. However, the molecular mechanism underlying autophagy in shrimp is still largely unknown. Previously, transcriptomes of crustaceans have been analyzed using traditional molecular technologies, such as cDNA microarray [19], suppression subtractive hybridization (SSH) [20], expressed sequence tag (EST) [21], and serial analysis of gene expression (SAGE) [22]. But each method has its inherent limitations of each, such as requirements for existing sequence information, background levels results from cross hybridization, time-

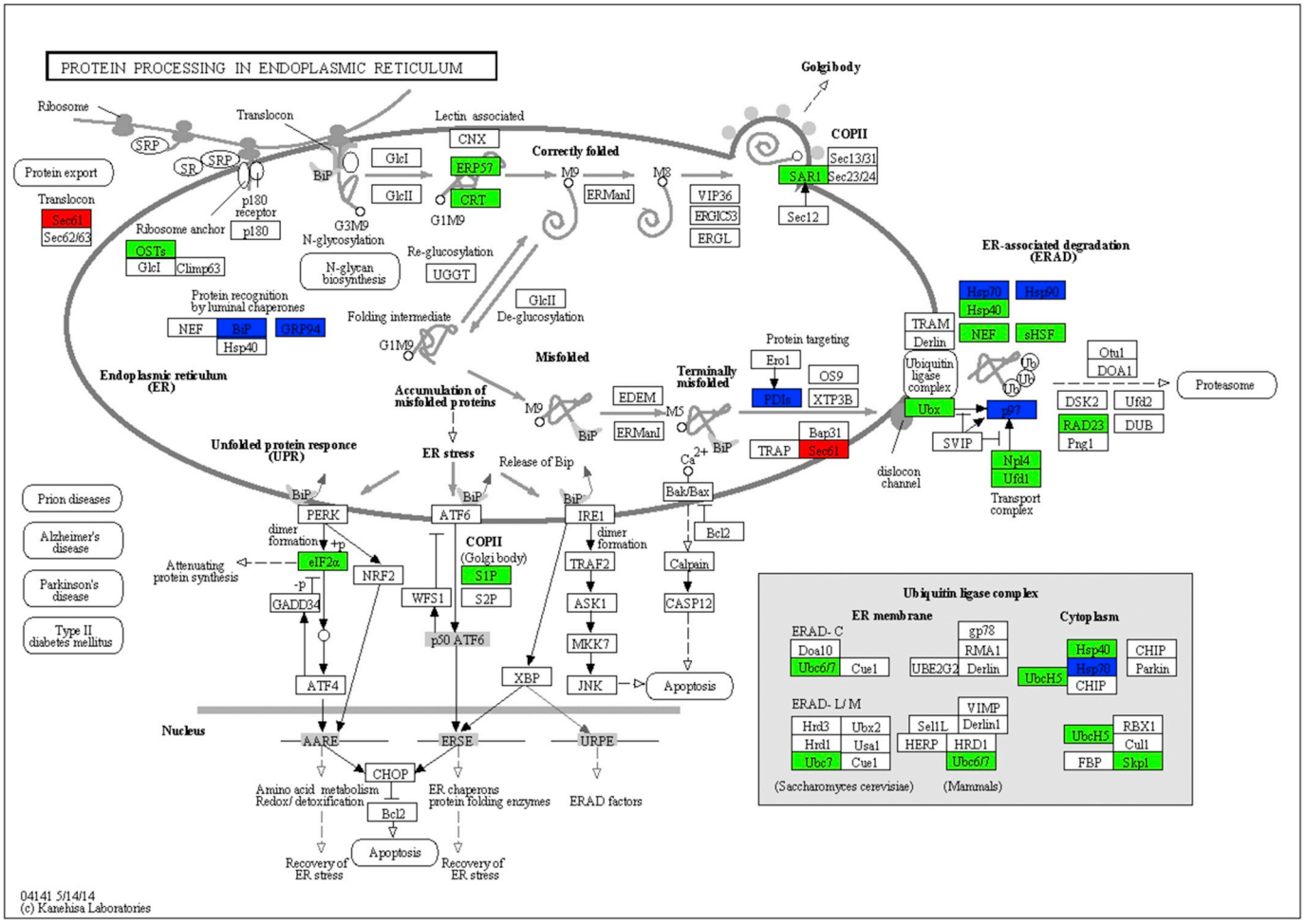

Fig. 7. KEGG pathway of protein processing in endoplasmic reticulum. Components with homologues in the *Litopenaeus vannamei* transcriptome are highlighted in green (down-regulated), red (up-regualted), and blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

consuming, costly, and cloning biases which have restricted their application in transcriptome analysis. Recently, the appearance of RNA-seq, a revolutionary tool for transcriptome profiling provides fascinating opportunities to resolve these problems. Compared with traditional methods, RNA-seq is more ideal method for transcriptome analysis due to its high efficiency, low cost, massive data output, high levels of reproducibility, and large dynamic range of gene expression profile [23].

In our present study, RNA-seq was employed to profile the transcriptome of *L. vannamei* treated with rapamycin. A total of 62,503 unigenes were yield from 89,847,358 clean reads which is comparable with previous studies [9,24]. The large-scale data will generate more valuable information for further investigation of new functional genes in shrimp. The mean length of unigenes is 835.9 bp (N50 = 1136 bp) which is much longer than 396 bp (N50 = 478 bp), 520 bp (N50 = 745bp) and 561 bp (N50 = 736) as previously reported [24–26]. The differences in sequence quality is probably resulted from the different sample size and tissues in previous studies. RNA for sequencing in previous studies was isolated from whole body of shrimp larvae, hemocytes or hepatopancreas, while our sample was prepared from gill cells which has less metabolic activity [8].

In addition, DEGs between the control and rapamycin-challenged group were analyzed by EBSeq method which could be used to compare digital gene expression profiles without biological repeats [16]. EBSeq analysis identified 3050 DEGs, including 1456 up-regulated and 1594 down-regulated genes. Functional annotation of the DEGs by matching to GO, COG and KEGG databases generates plenty of unigenes which contribute to our understanding of the biological features of shrimp in response to rapamycin challenge. There are 34 unigenes classified into “PI3K-Akt signaling pathway” which affects autophagy. In all eukaryotes, the mTOR signaling pathway couples energy and nutrient abundance to execute cell growth and division. As a highly conserved kinase in the PI3K family, mTOR leads to a swift response to various environmental cues, regulating cell metabolism and immune responses by regulating the kinase Akt. Activation of the PI3K-AKT-mTOR signaling pathway inhibits the autophagy. There are 50

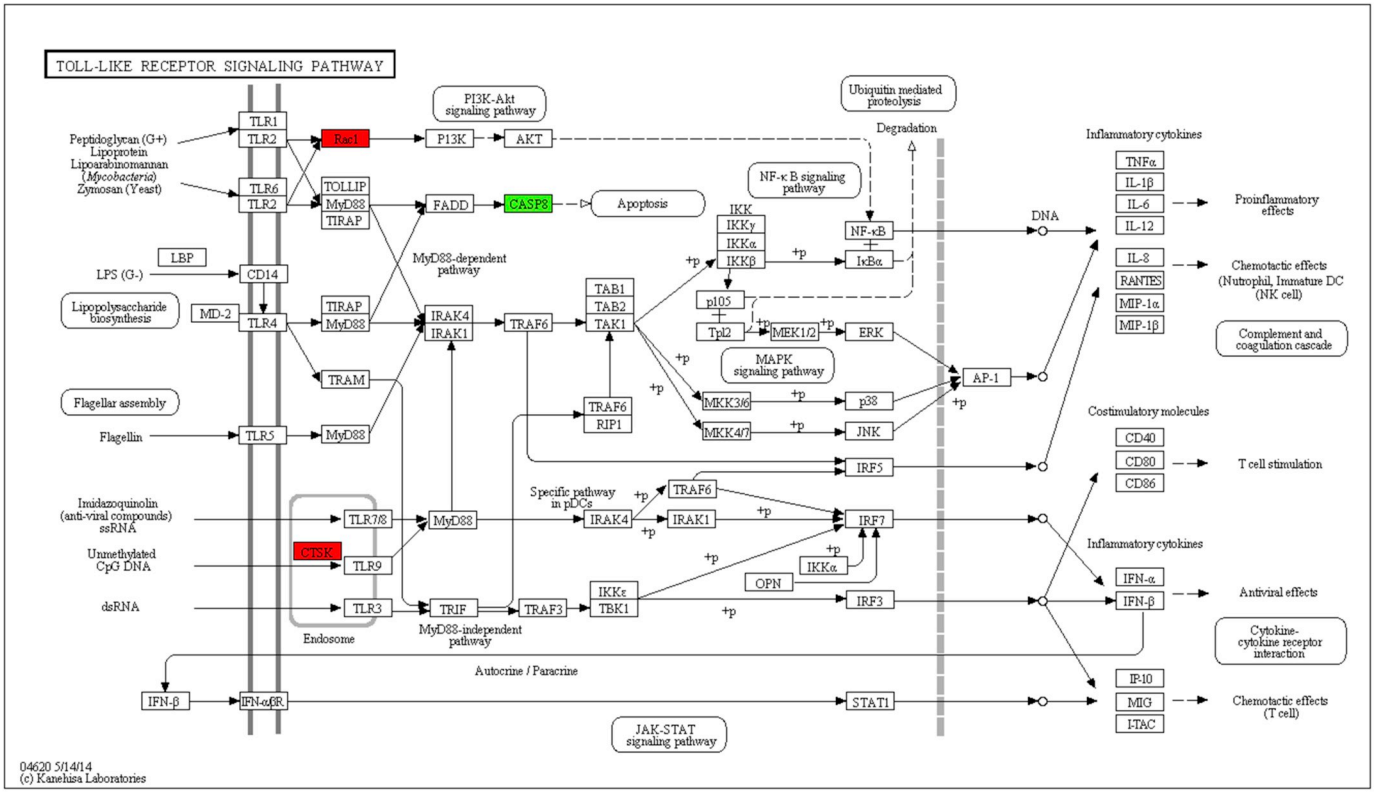

Fig. 8. Toll-like receptor signalling pathway coverage. The Toll-like receptor signalling pathway in the KEGG database. Proteins in the pathway are depicted by boxes while arrows depict signalling routes. Pathway components with homologues in the *Litopenaeus vannamei* transcriptome are highlighted in red (up-regulated) and green (down-regulated). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

unigenes classified into “lysosome” KEGG pathway which is closely associated with autophagy. Lysosomes play important functions in maintaining metabolic homeostasis by removing cellular material of the endocytic and autophagic pathways [27,28]. During autophagy, autophagosomes fuse with lysosomes to form autolysosomes which enable the degradation of unwanted cytoplasmic material [29]. Phagocytosis is an essential defense mechanism of innate immunity, by recognizing, engulfing and destroying invading microbes. Acceleration of phagosome maturation enhances pathogen killing, while a delay in phagosome maturation preserves antigenic peptides for presentation to T cells and to initiate adaptive immune responses. Immune signals, such as pathogen-associated molecular patterns (PAMPs), accelerates phagosome maturation to enhance pathogen killing [30]. Our results showed that 57 unigenes are classified into “phagosome” KEGG pathway and 43 unigenes are classified into “antigen processing and presentation” KEGG pathway in response to rapacymin induction, indicating that autophagy may affect shrimp innate immunity. More importantly, Toll-like receptors (TLRs) signaling pathway and NF-kB signaling pathway were founded in KEGG pathway. Toll-like receptors (TLRs) are one of the primary mediators of the innate immune system in animals from insects to humans. TLRs recognize specific microbial pathogen molecules and initiate intracellular signaling cascades to activate a variety of transcription factors, including NF-κB, which then direct the expression of key innate immune effector genes participating in immunity, inflammation, cell proliferation and cell death. Tolls identified in shrimp *Litopenaeus vannamei* [31], *Fenneropenaeus chinensis* [32], *Penaeus monodon* [33], and *Marsupenaeus japonicus* [34] play important roles in shrimp innate immunity. LvToll1–3 responded to *Vibrio alginolyticus* and WSSV infections in *L. vannamei* [31]. FcToll and MjToll respond to various immune challenges [32]. LvToll in *L. vannamei* regulates AMP expression after challenge with *V. anguillarum* and *Micrococcus lysodeikticus* [31].

In summary, the present study firstly reported the identification of important genes in response to rapamycin challenge at transcriptional level in shrimp *L. vannamei*. We found that a total of 3050 unigenes are differentially expressed. Interestingly, among them, 57 unigenes classified into “phagosome”, 34 unigenes classified into “PI3K-Akt signaling pathway”, 43 unigenes classified into “antigen processing and presentation”, and 50 genes classified into“lysosome” KEGG pathways are regulated in response to rapacymin induction, indicating that autophagy may affect shrimp innate immunity.

Our findings will contribute to our understanding of the molecular mechanism underlying autophagy. In the future, further studies will be performed to investigate the functions of these DEGs in autophagy in shrimp and other crustaceans.

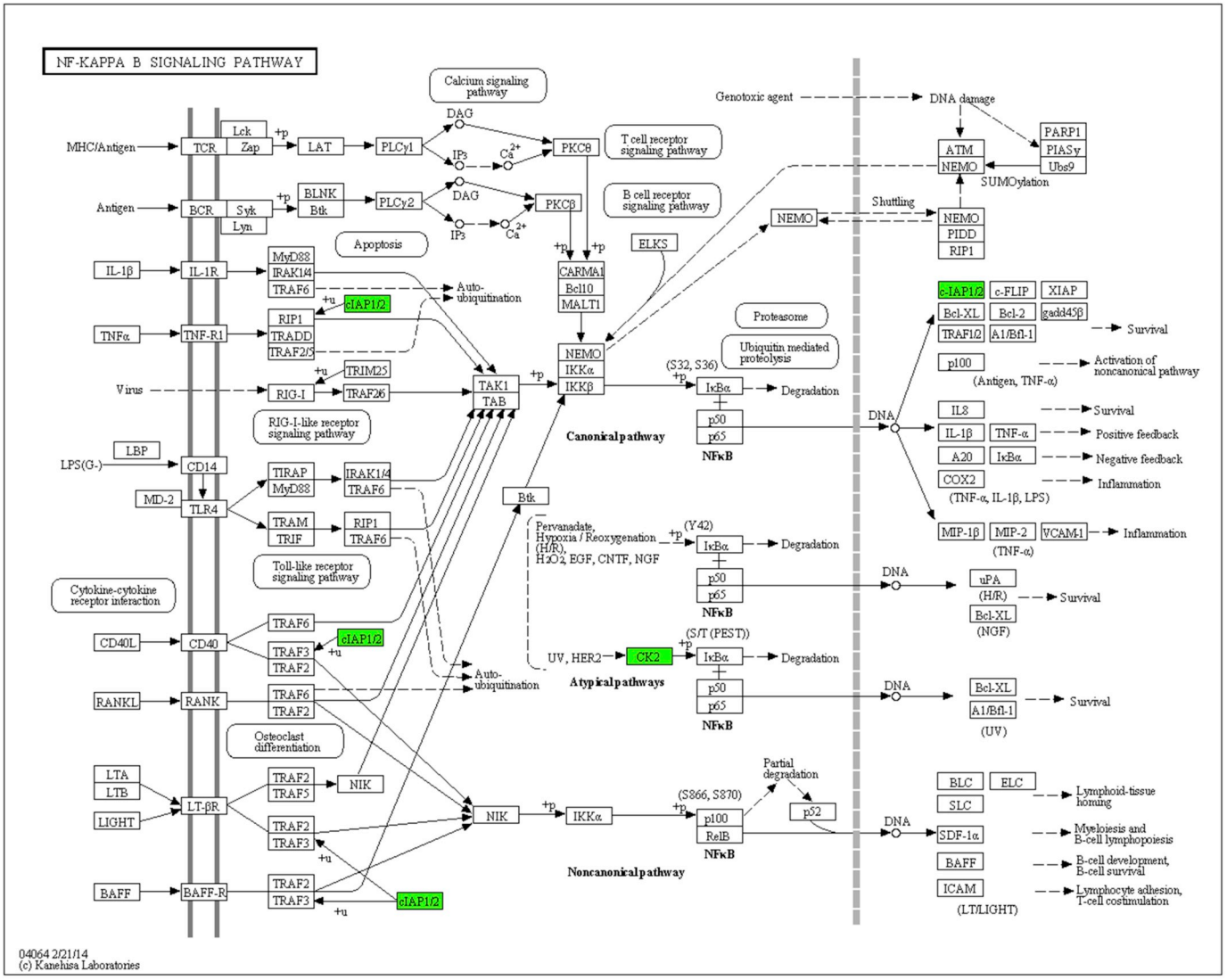

Fig. 9. NF-kB signalling pathway. Overview of the KEGG NF-kB signalling pathway. Components with homologues in the *Litopenaeus vannamei* transcriptome are highlighted in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Declarations of interest

None

Acknowledgments

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