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RNA-Seq analysis reveals genes associated with resistance to Taura syndrome virus (TSV) in the Pacific white shrimp *Litopenaeus vannamei*

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ABSTRACT

Outbreak of Taura syndrome virus (TSV) is one of the major pathogens of the Pacific white shrimp *Litopenaeus vannamei*. Although selective breeding for improvement of TSV resistance in *L. vannamei* has been successfully developed and has led to a great benefit to the shrimp farming industry worldwide. The molecular mechanisms underlying the viral resistance in shrimp remain largely unknown. In the present study, we conducted the first transcriptomic profiling of host responses in hemolymph and hemocytes in order to identify the differentially expressed genes associated with resistance to TSV in *L. vannamei*. High-throughput RNA-Seq was employed, obtaining 193.6 and 171.2 million high-quality Illumina reads from TSV-resistant and susceptible *L. vannamei* lines respectively. A total of 61,937 contigs were generated with an average length of 546.26 bp. BLASTX-based gene annotation (E-value < 10^{-5}) allowed the identification of 12,398 unique proteins against the NCBI non-redundant NR database. In addition, comparison of digital gene expression between resistant and susceptible strains revealed 1374 significantly differentially expressed contigs (representing 697 unigenes). Gene pathway analysis of the differentially expressed gene set highlighted several putative genes involved in the immune response activity including (1) pathogen/antigen recognition including immune regulator, adhesive protein and signal transducer; (2) coagulation; (3) proPO pathway cascade; (4) antioxidation; and (5) protease. The expression patterns of 22 differentially expressed genes involving immune response were validated by quantitative real-time RT-PCR (average correlation coefficients 0.94, p-value < 0.001). Our results provide valuable information on gene functions associated with resistance to TSV in *L. vannamei*.

1. Introduction

Shrimp are globally important aquaculture species cultivated in tropical and sub-tropical waters (Bailey-Brock, 1992), primarily in Asia and the Americas. However, the shrimp industry is facing serious challenges due to disease problems that cause significant economic losses (Moss et al., 2001, 2007, 2012; Argue et al., 2002). Among many of the disease problems, viral diseases are the leading causes of economic losses.

Back in the 1990s, TSV was among the most serious viral pathogens for the shrimp industry (Overstreet et al., 1997; OIE, 2002). TSV disease outbreaks caused major damages to shrimp populations. Such damages were extremely severe with the Pacific white shrimp, *L. vannamei* due to the very high mortality rate, ranging from 40% to 100% (Jimenez, 1992). Since mid-1990s, great efforts have been devoted to controlling TSV disease through selective breeding of disease resistance (Moss et al., 2012). Because of such efforts of selective breeding, the outbreaks of Taura syndrome have been significantly reduced with the TSV resistant shrimp lines.

In the last decade, good progress has been made for the generation of genomic resources for the dissection of genomic structure, organization and analysis of performance traits-related genes in crustacean (Andriantahina et al., 2013). Such progress included the development of molecular markers (Leu et al., 2011; Duan et al., 2013), generation of large-insert BAC libraries (Zhang et al., 2010, 2011), construction of genetic and physical maps, and efforts for sequencing the entire genome of the penaeid shrimp (Shen et al., 2007; Du et al., 2010; You et al., 2010; Lin et al., 2012). These genomic resources made it possible to address the issues of disease resistance at the genome and transcriptome levels using various approaches such as microarrays (Zeng and Lu, 2009; Aoki et al., 2011). For instance, with white spot syndrome

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virus (WSSV), a set of host response genes were identified. Wang et al. (2006) reported the identification of the genes in response to WSSV infection in *Fenneropenaeus chinensis* through cDNA microarray technology (Wang et al., 2006a). Novel white shrimp specific genes and the genes involved in stress and immune response were identified from WSSV-infected shrimp gill cDNA library as well (Clavero-Salas et al., 2007). A large number of conserved genes were identified to take part in the immune responses in shrimp host-virus interactions (Robalino et al., 2007). In addition, study focusing on the shrimp molecular responses to the viral pathogens were performed (Sritunyalucksana et al., 2012). The sequence diversity, expression, gene structure, and antimicrobial activities of cationic antimicrobial peptides (AMPs) in penaeid shrimps were discussed recently (Tassanakajon et al., 2011). Three novel WSSV recognizing C-type lectins (CTLs) were cloned and characterized in essential roles in shrimp *Marsupenaeus japonicas*, indicating their role in innate immune responses and the shrimp molecular defense system to viral pathogens (Song et al., 2010). Characterization of the virus-binding proteins has revealed their roles in shrimp innate immunity and their potential therapeutic applications to the shrimp aquaculture industry (Sritunyalucksana et al., 2012). A comprehensive *L. vannamei* transcriptome of a relatively high-WSSV-resistant shrimp family containing more than 73,000 annotated unigenes was generated using Illumina high-throughput sequencing, which greatly enriched the transcriptomic resources (Li et al., 2012a). Differences of the shrimp transcriptome at WSSV Li stage and AI stage were identified by Illumina sequencing. Many WSSV-responded genes and pathways were revealed in host defense against WSSV acute infection (Li et al., 2013).

Years of studies allowed the identification of several pattern recognition receptors, such as lectin, toll, lipopolysaccharide and β-1,3-glucan binding protein (LGBP) and tetraspanin. Primary gene pathways that are putatively involved in the immune response of shrimp were identified (Li and Xiang, 2013). The discovery and functional characterization of the immune molecules in penaeid shrimps were well described, in which the molecules contributing to the immune defense were emphasized (Tassanakajon et al., 2013). In addition, many studies have been carried out to better understand how shrimps respond to viral infections. However, most studies were conducted with WSSV, studies with TSV have been limited despite the success of selective breeding for TSV resistant lines. Transcriptomic analysis using a cDNA microarray containing 2469 putative unigenes were performed to gain insight into the response to viral infection of two strains of the Pacific white shrimp (*L. vannamei*) that differ in their resistance to TSV. The results revealed the possible transcriptomic signatures of protein metabolism, cellular trafficking, immune defense and stress response in shrimp against TSV (Veloso et al., 2011). Recent studies about the interaction between shrimp immunity and TSV infection was conducted using 454 pyrosequencing. The hepatopancreas transcriptome of *L. vannamei* was sequenced and assembled, and a set of genes were identified to be significantly differentially expressed with the potential molecular functions, such as antiviral, antimicrobial, proteases, protease inhibitors, signal transduction, transcriptional control, cell death and adhesion. However, their associated gene pathways remain largely unknown (Zeng et al., 2013).

To gain knowledge on TSV resistance in shrimp, we conducted RNA-Seq analysis using TSV- resistant and susceptible *L. vannamei* lines. The transcriptional profiles of the hemocyte between TSV-resistant and susceptible *L. vannamei* lines were compared and analyzed. Here we report a set of 697 significantly differentially expressed genes with critical roles in immune function including pathogen recognition, coagulation, proPO pathway, antioxidation, and protease.

2. Materials and methods

2.1. Shrimp sample

The TSV-resistant and susceptible *L. vannamei*, reared under pathogen-free conditions were obtained from SyAqua Siam Co. Ltd. The two shrimp lines exhibited significant differences in survival rate when challenged with TSV, *i.e.*, the resistant shrimp showed 60–80% survival whereas the susceptible line had only 20% survival (unpublished data). The shrimps were acclimatized in laboratory tanks continuously supplied with constant temperature and flow-through sea water at 20 °C, 20 parts per thousand (ppt) salinity and continuous aeration for 7 days before use in the experiments.

2.2. Hemocyte collection and RNA extraction

Hemolymph of TSV-resistant and susceptible shrimps was drawn from the ventral sinus using a sterile 1 ml syringe with 150 μl anticoagulant [10% (w/v) sodium citrate]. The hemolymph was centrifuged immediately at 5000*g* for 5 min at 4 °C to separate the hemocytes from plasma. The hemocytes from each group were separately preserved in liquid nitrogen immediately for RNA extraction. Total RNA was isolated using TRI Reagent® (Molecular Research Center, Inc.) according to the instructions provided by the manufacturer and then incubated with RNase-free DNase I (Promega) to eliminate genomic DNA contamination. Total RNA concentration and integrity was assessed by an Agilent 2100 Bioanalyzer using a RNA Nano Bioanalysis chip. For each group, equal amount of RNA from the eight shrimp individuals per line were pooled to provide templates for RNA-Seq library construction.

2.3. Library construction and Illumina sequencing

RNA-Seq library preparation and sequencing was outsourced and performed in the Genomic Services Lab of the HudsonAlpha Biotechnology Institute (Huntsville, AL, USA) as previously described (Li et al., 2012b; Sun et al., 2012). The cDNA libraries of TSV-resistant and susceptible shrimp were separately synthesized by 2–4 μg of starting DNase-treated total RNA following the protocols of the Illumina TruSeq RNA Sample Preparation Kit (Illumina) as described in the TruSeq protocol. The libraries were amplified with 15 cycles of PCR and contained TruSeq indexes within the adaptors, barcoded for the resistance and the susceptible lines. The final libraries had an average fragment size of ~270 bp and final yields of ~400 ng. After KAPA quantitation and dilution, the libraries were sequenced using a single lane on an Illumina HiSeq 2000 instrument with 100 bp paired end (PE) reads.

2.4. De novo assembly and transcriptome analysis

The high-quality sequences were required for *de novo* assembly analysis. Before assembly, raw sequencing reads were trimmed by removing adapter sequences and ambiguous nucleotides. Sequencing reads with quality scores less than 20 (low quality sequence) and read length below 30 bp (short read length sequence) were all trimmed using CLC Genomics Workbench (version 4.8; CLC bio, Aarhus, Denmark). The remaining high-quality reads were used in the subsequent assembly. *De Brujin* graph based assemblers such as ABySS and Trans-ABySS were considered to produce an optimized assembly results. Firstly, the sequence reads were *de novo* assembly using the ABySS program version 1.3.4 (Simpson et al., 2009) and Trans-ABySS program version 1.3.2. These programs were used to reduce the dataset into smaller sets of

non-redundant contigs and to generate a *de novo* transcriptome (Robertson et al., 2010). ABySS assembly was performed at various k-mer lengths from 50 to 96, assemblies from all k-mers were merged into one transcriptome assembly by Trans-ABySS. The redundant contigs and singletons were removed using CAP3 (Huang and Madan, 1999). The resulting contigs (final non-redundant transcripts) that were >200 bp were retained as a reference assembly for subsequent analysis.

2.5. Transcriptome annotation and gene ontology

The NCBI *Tribolium castaneum* protein database and the non-redundant (nr) protein database were downloaded and used as reference protein database. For analysis, the assembled contigs were used as queries for BLAST searches against the reference protein databases. BLASTX searches were conducted with an *E*-value cut-off of 1e-5. The top hits were parsed into Excel file for further analysis. Gene ontology (GO) annotation analysis was carried out using the BLAST results in Blast2GO version 2.5.0 (http://www.blast2go.org/). The final annotation output was produced after gene ID mapping, GO term assignment, annotation augmentation and generic GO-Slim process and then the annotation result was analyzed with respect to cellular component, biological process, and molecular function as we previously reported (Li et al., 2012b; Sun et al., 2012).

2.6. Analysis of differentially expressed contigs

High-quality reads were used for further analysis using "RNA-Seq Analysis" and "Gene Expression Analysis modules of the CLC Genomics Workbench package. First, the reads were mapped onto Trans-ABySS reference assembly using CLC Genomics Workbench software version 4.8.1. During mapping, the parameters were set to 95% to align to the reference and a maximum of two mismatches were allowed. The total mapped reads results for each transcript were analyzed, and then normalized to RPKM to account for the difference in transcript length (Reads Per Kilobase of exon model per Million mapped reads). The proportions-based test was used to identify the differently expressed genes between TSV-resistant and susceptible lines with p-value < 0.05. The proportions-based test protocol was originally developed for SAGE data which allows an estimation of differential expression based on single measurements of tag/read counts for two conditions (Kal et al., 1999). After normalization of the RPKM values, fold changes between TSV-resistant and susceptible shrimp lines were calculated. Differential gene expression analysis was carried out by using the RNA-Seq module and the expression analysis module in CLC Genomics Workbench. Transcripts with a minimal reads of 10 were used for analysis of differential expressed genes, and fold change values of transcripts of larger than 1.5 and were included as differently expressed genes.

Gene pathway analysis of significantly differentially expressed genes was carried out based on GO analysis, pathway analysis, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database as well as manual literature review.

2.7. GO term enrichment analysis

To identify overrepresented GO annotations in the differentially expressed gene sets as compared to the broader reference assembly, statistical GO analysis was performed using Ontologizer 2.0 (Bauer et al., 2008). The analysis was conducted using parent–child intersection method with a Benjamini–Hochberg correction (Grossmann et al., 2007) for multiple testing correction. The 'study set' corresponded to the frequency of GO terms in the differentially expressed genes set, while the 'population set' indicated the whole shrimp hemocyte transcriptome.

2.8. Confirmation using quantitative real-time RT-PCR (QPCR)

To examine the reliability of the RNA-Seq results, a selected subset of differentially expressed genes involved in immune responses were selected for validation using quantitative real-time RT-PCR (QPCR). The hemocytes from TSV-resistant and susceptible lines from SyAqua Siam Co.Ltd were used as the template for RNA extraction using the TRI Reagent® (Molecular Research Center, Inc.), and then treated with RQ1 RNase-free DNase (Promega) to remove genomic DNA contamination. The master-pooled samples were created by combining equal amounts of RNA from three replicate pools (three individual shrimp per pool), to assess expression profiles. The first stand cDNA was synthesized from 1 μg of DNase-treated total RNA by RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. The cDNAs were amplified by PCR using specific primers designed based on RNA-Seq contig sequences (Supplementary Table 1) using SECentral Primer Designer program version 4.20 and Primer3 program version 0.4.0. All the cDNAs templates were diluted to 250 ng/μl and utilized for the QPCR using the SsoFast™ EvaGreen® Supermix. The thermal cycling profile consisted of an initial denaturation at 95 °C (for 30 s), followed by 40 cycles of denaturation at 94 °C (5 s), an appropriate annealing/extension temperature (58 °C, 5 s). An additional temperature ramping step was utilized to produce melting-curves of the reaction from 65 °C to 95 °C. The housekeeping gene β-actin was used as the reference gene. Relative fold changes were analyzed in the mathematical model of the Relative Expression Software Tool version 2009 based on the cycle threshold (Ct) values generated by QPCR (Pfaffl et al., 2002). The QPCR was performed in triplicate on a CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Expression differences between TSV-resistant and susceptible lines were calculated for statistical significance using a randomization test in the REST software. Expression levels of β-actin gene were constant between all samples (<0.30 change in Ct). A no-template control was run on all plates. The differences in expression were analyzed using one-way analysis of variance (ANOVA) (Steel and Torrie, 1980). A multiple-comparison (Duncan's) test was used to examine significant differences among treatments using SPSS software (Duncan, 1955). Statistical significance of differences was set at $p < 0.05$.

3. Results

3.1. Sequencing of short expressed reads from L. vannamei hemocyte

Illumina-based RNA-Sequencing (RNA-Seq) was conducted with samples from TSV-resistant and susceptible *L. vannamei* lines. A total of 378.35 million of 100 bp paired end (PE) reads were generated. Over 177 million reads were generated for each of the two libraries. After trimming of low-quality reads (quality scores < 20) and short reads sequences (less than 30 bp), a total of 364.83 million high-quality sequences (96.4%) were obtained (Table 1), and these sequences were carried forward for additional analysis. All the sequences with raw read data were deposited at the NCBI Sequence Read Archive (SRA) under Accession SRP018120.

3.2. De novo assembly of the transcriptome

Assembly of the 364.83 million short reads using of ABySS and Trans-ABySS resulted in approximately 2.6 million contigs with N50 size of 689 bp. Following removal of redundancy using CAP3,

Table 1
Summary of Illumina expressed short reads production and filtering. Pooled hemocyte samples from TSV-resistant and susceptible shrimp lines were used for RNA preparation. Paired-end reads were generated on an Illumina HiSeq 2000 instrument.

	TSV-resistant shrimp	TSV-susceptible shrimp
No. of reads	201,064,238	177,287,468
Average read length (bp)	100	100
Number of reads after trimming	193,608,168	171,229,468
Percentage retained	96.29%	96.58%
Average read length after trimming (bp)	90.9	90.9

Table 2
Significantly differentially expressed contigs between the hemocyte from TSV-resistant and susceptible *L. vannamei* with BLAST search databases ($p < 0.05$, *E*-value $\leqslant$1e−5, fold change ~1.5). The TSV- susceptible shrimp group was used as the control group.

	No. of differentially expressed contigs	No. of unique genes
Up-regulated in resistant line	877	483
Down-regulated in resistant line	497	214
Total	1,374	697

a total of 61,937 contigs with an average length of 546 bp were used as the reference of *L. vannamei* hemocyte transcriptome for annotation and analysis of differentially expression gene profiling.

3.3. L. vannamei hemocyte transcriptome annotation

BLAST searches were performed for the annotation of the *L. vannamei* hemocyte transcriptome. The contigs assembled by the ABySS and Trans-ABySS programs were used as queries in BLASTX searches against the NCBI *T. castaneum* protein database and the nr databases. A total of 9609 assembled contigs had significant (*E*-value $\leqslant$1e−5) hits against the NCBI *T. castaneum* protein database, representing 4718 unique proteins. When searching against the nr databases, the assembled contigs corresponded to a total of 12,398 unique proteins (Supplementary Table 2).

After initial BLAST searches, BLAST2GO analysis was conducted to categorize the known genes into level 2 functional groups. As shown in Fig. 1, the largest percentage of known genes was categorized into cellular component and molecular function. Of the Molecular Function category, binding and catalytic activity accounted for the largest number of genes. A total of 5082 GO terms were assigned to the unique gene matches, including 1682 (33%) cellular component terms, 1403 (28%) molecular functions terms and 1997 (39%) biological process terms. Analysis of level 2 GO term distribution showed that cell (GO:0005623), binding (GO:0005488), catalytic activity (GO:0003824), and metabolic process (GO:0008152) were the most common annotation terms in the three GO categories (Fig. 1).

3.4. Differentially expressed genes between the TSV-resistant and susceptible L. vannamei lines

A total of 1374 of the 61,937 (2.22%) final assembled contigs demonstrated significant differentially expressed profile between TSV-resistant and susceptible *L. vannamei* (Supplementary Table 3). Of the 1374 contigs that exhibited differential expression profiles, 877 were up-regulated in the resistant line, and 497 were down-regulated in the resistant line, as compared with the expression profiles in the susceptible line. The 877 up-regulated contigs represented 483 unique genes, and the 497 down-regulated contigs represented 214 unique genes. Taken together, the 1374 differentially expressed contigs represented a total of 697 known genes that are differentially expressed between the resistant and the susceptible lines (Table 2).

3.5. Enrichment and pathway analysis

The differentially expressed genes were used to perform GO-term enrichment analysis. Parent–child GO term enrichment analysis detected a total of 72 significantly overrepresented GO terms were enriched in response to TSV infection, with FDR-corrected p-value < 0.1. Among these, 10 higher level GO terms were retained as informative (Table 3), including response to stimulus (GO:0050896), proteolysis (GO:0006508), immune response (GO:0006955), positive chemotaxis (GO:0050918), antigen processing and presentation (GO:0019882), response to stress (GO:0006950), regulation of cell death (GO:0010941), structural

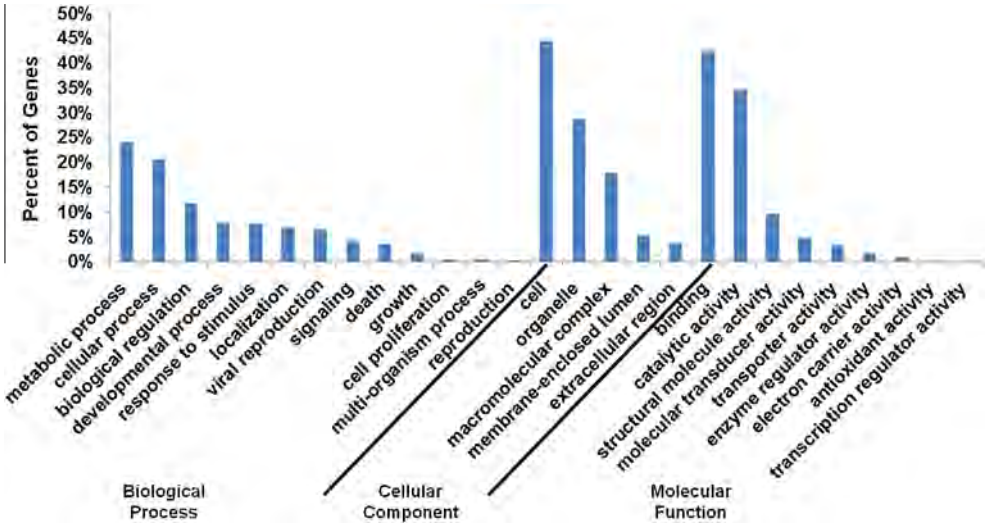

Fig. 1. Gene ontology (GO) term categorization and distribution of assembled Trans-ABySS contigs encoding genes. GO-terms were processed by Blast2Go and categorized at level 2 under three main categories (cellular component, molecular function and biological process).

Table 3
Summary of GO term enrichment result of significantly expressed genes between TSV-resistant and susceptible shrimp group. Count = study count/population count where population count is the number of genes associated with the term in the population set and study count is the number of genes associated with the term in the study set.

GO ID	Name	Adjusted *P*-value	Count
GO:0050896	Response to stimulus	5.31E-09	45/106
GO:0006508	Proteolysis	7.51E-05	29/43
GO:0006955	Immune response	8.74E-03	69/134
GO:0050918	Positive chemotaxis	3.06E-02	54/103
GO:0019882	Antigen processing and presentation	2.89E-02	29/43
GO:0006950	Response to stress	0.000202	52/163
GO:0010941	Regulation of cell death	0.000716	17/45
GO:0005198	Structural molecule activity	0.00378	26/93
GO:0030029	Actin filament-based process	0.00467	41/182
GO:0009607	Response to biotic stimulus	0.0073	12/37

molecule activity (GO:0005198), actin filament-based process (GO:0030029), and response to biotic stimulus (GO:0009607).

Due to the incomplete assembly and limited annotation of the shrimp genome, therefore, we used a combination of KEGG pathway analysis, manual re-annotation based on the NCBI *T. castaneum* protein database and the nr database, and manual literature searches to identify broad functional categories of the differentially expressed genes. As listed in Table 4, five functional categories were identified to potentially play important roles associated with TSV infection including (1) pathogen/antigen recognition including immune regulator, adhesive protein and signal transducer; (2) coagulation; (3) proPO pathway cascade; (4) antioxidation; and (5) protease (Fig. 2). Putative functional roles and interactions of these genes involved in mediating the shrimp response to heat stress are discussed in detail in Discussion.

3.6. Validation of differentially expressed genes by quantitative real-time PCR (QPCR)

QPCR was performed on 22 randomly selected genes for validating the differentially expressed genes identified by RNA-Seq. Purified total RNA of hemocyte from the TSV resistant and susceptible shrimp lines were used for generation the first stand cDNA. These cDNAs were used as the templates for QPCR. Primers were designed based on contig sequences (Supplementary Table 1). For the results, the melting-curve analysis of QPCR demonstrated a single product for all tested genes. Fold changes from QPCR were compared with the RNA-Seq expression profiles. As shown in Fig. 3, most of QPCR results were significantly correlated with the RNA-Seq results (correlation coefficients 0.94, p-value < 0.001). In general, QPCR analysis was used for confirmation of the RNA-Seq results, indicating the reliability and accuracy of the Trans-ABySS reference assembly and RNA-Seq expression analysis.

4. Discussion

Viruses such as TSV have been proven to infect and cause mortality and morbidity in Penaeid shrimp. Because of the huge economic losses of TSV on commercial shrimp farming around the world, the TSV-resistant domesticated stocks of shrimp (*L. vannamei and Litopenaeus stylirostris*) have been successfully developed. The resistant line exhibited a 60–80% survival rate whereas the susceptible line had only 20% survival rate. However, the molecular mechanisms underlying resistance to TSV in shrimp is still largely unknown. As the first attempt to understand the molecular basis for the viral resistance in shrimp hemocyte, in this study we conducted a high throughput RNA-Seq analysis using Illumina next generation sequencing. Such high throughput RNA-Seq analysis allowed us to identify a large number (697) of differentially expressed genes between the resistant and the susceptible lines.

RNA-Seq analysis for transcriptome profiling has been proved to be a robust and reliable approach to assess transcriptional responses to various experimental conditions, such as disease resistance, heat stress and hypoxia. A compromise in this kind of study is the use of master-pooled samples, created by combining equal amounts of RNA from three replicate pools (three individual shrimp per pool in the present study). To a certain extent, this economical approach probably masked some degree of individual variation in transcript levels among individual shrimp. However, to gain a broad understanding and provide the early insights into important functional pathways and processes in the shrimp TSV hemocyte, the master-pooled samples method was employed. Follow-up studies will be more targeted on the variations among different individual. The validation experiment was performed using QPCR, examining whether the expression pattern of the differentially expressed genes in RNA-Seq matched what we observed in QPCR. Differentially expressed genes with various expression patterns from both TSV-resistant and susceptible lines were tested. Significant correlations between the QPCR and RNA-Seq was observed ($R = 0.94$, p-value < 0.001), which indicated the reliability of RNA-Seq for gene expression analysis in this study (Fig. 3). The difference between the two techniques was only observed in a few genes (chitinase, serpin, Masq and C-type lectin in Fig. 3) which exhibit slight fold change between the two groups. This might be due to differences in expression between replicate pools or inaccurate quantification by RNA-seq.

Although selective breeding program has been beneficial to resist TSV (Argue et al., 2002), this strategy has not been effective for other viral pathogens. Recently, the transcriptome of TSV resistant and susceptible *L. vannamei* when infected with TSV and YHV were investigated and changes in gene expression in the hepatopancreas were assessed using a cDNA microarray (Veloso et al., 2011). The study reported that many genes were differentially expressed in hepatopancrease of susceptible and resistant *L. vannamei*. Although some immune genes such as fortilin, crustin, penaedin etc. were up-regulated in the resistant strain, immune pathways responsible for the TSV resistance could not be clearly demonstrated. Interestingly, they revealed that some energy and growth-related genes were down-regulated in the TSV resistant strain which is consistent with the results of Argue et al. (2002) who reported a negative correlation between growth and TSV resistance. In contrast to our results, most genes in three main categories (immune-related genes, molecular function and biological process) were up-regulated in the TSV resistant strain (Table 4, Supplementary Table 4). The different findings might result from different tissues examined. Hemocytes and hemolymph are the main sites where several immune reactions take place while hepatopancreas plays a major function in digestion and detoxification. Therefore, changes in

Table 4
Differentially expressed immune-related genes between TSV-resistant and susceptible *L. vannamei* lines.

Accession No.	Gene name	Contig ID	Fold change
Pathogen recognition			
Immune regulator			
XP_003627732.1	ATP synthase subunit beta [*Medicago truncatula*]	2727553	−9.07
ABR23368.1	fibrinogen domain-containing salivary secreted protein [*Ornithodoros parkeri*]	1058922	−3.52
AEM76723.1	fibrinogen-related protein 1 [*Marsupenaeus japonicus*]	152054	−3.11
AEH05998.1	C type lectin containing domain protein [*Litopenaeus vannamei*]	529127	−1.53
ADQ43366.1	HMGBa [*Litopenaeus vannamei*]	852481	2.05
XP_003588326.1	ATP synthase subunit alpha [*Medicago truncatula*]	2724814	2.67
Adhesive protein			
ADW79421.1	peroxinectin [*Procambarus clarkii*]	1006305	−2.21
EGI59793.1	integrin alpha-PS2 [*Acromyrmex echinatior*]	1244868	3.02
EGI68536.1	integral membrane protein [*Acromyrmex echinatior*]	2725731	5.56
Signal transducer			
BAJ41257.1	kruppel homolog 1 isoform A [*Frankliniella occidentalis*]	2268688	−29.86
AEW22981.1	kruppel-like protein 1 [*Thermobia domestica*]	2727164	−21.26
XP_003706092.1	serine/threonine-protein phosphatase 2A activator-like [*Megachile rotundata*]	2707166	−6.4
XP_001663511.1	receptor protein tyrosine phosphatase, putative [*Aedes aegypti*]	269091	−5.55
EHJ65114.1	putative zinc finger protein [*Danaus plexippus*]	103360	1.51
EFN83844.1	MAP kinase-interacting serine/threonine-protein kinase 1 [*Harpegnathos saltator*]	2727571	1.52
EFN63843.1	rab11 family-interacting protein 1 [*Camponotus floridanus*]	2394384	1.53
XP_001602345.2	serine/threonine-protein kinase mTOR [*Nasonia vitripennis*]	1520489	1.54
XP_003705177.1	probable Ras GTPase-activating protein-like [*Megachile rotundata*]	2732112	1.55
XP_003738175.1	serine/threonine-protein kinase RIO2-like [*Metaseiulus occidentalis*]	364535	1.58
ACY66411.1	map kinase-interacting serine/threonine [*Scylla paramamosain*]	2713223	1.58
AEK86516.1	toll2 [*Litopenaeus vannamei*]	2502719	1.58
ABK57056.1	*n*-myristoyltransferase, putative [*Glyptapanteles indiensis*]	935198	1.62
AFK65508.1	cyclin-dependent kinases 2 [*Macrobrachium rosenbergii*]	2725451	1.64
XP_002425169.1	diacylglycerol kinase zeta, putative [*Pediculus humanus corporis*]	2502840	1.65
EFX74605.1	shn zinc finger protein [*Daphnia pulex*]	2733200	1.72
EKC36121.1	proto-oncogene tyrosine-protein kinase Src [*Crassostrea gigas*]	2727991	1.78
XP_524493.3	zinc finger protein 628 [*Pan troglodytes*]	1640185	1.86
BAI70368.1	suppressor of cytokine signaling-2 like protein [*Marsupenaeus japonicus*]	2731674	1.91
EGI69607.1	zinc finger protein 585B [*Acromyrmex echinatior*]	280747	1.91
XP_002428675.1	zinc finger protein FYVE domain-containing protein, putative [*Pediculus humanus corporis*]	1030144	1.92
XP_785013.3	serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit A-like[*Strongylocentrotus purpuratus*]	543829	2
XP_784091.2	zinc finger protein 658B-like [*Strongylocentrotus purpuratus*]	2730247	2.03
P27446.3	tyrosine-protein kinase Fyn[*Xiphophorus hellerii*]	270553	2.35
XP_001031872.1	protein kinase domain containing protein [*Tetrahymena thermophila*]	275730	3.25
XP_002432222.1	serine/threonine-protein kinase wnk 1,3,4, putative [*Pediculus humanus corporis*]	2725015	3.7
XP_003439683.1	ras-specific guanine nucleotide-releasing factor RalGPS1-like [*Oreochromis niloticus*]	1745161	13.1
XP_003492173.1	zinc finger and BTB domain-containing protein 37-like isoform 1 [*Bombus impatiens*]	2725296	27.53
Protease			
EGI70840.1	aminopeptidase N [*Acromyrmex echinatior*]	2727537	−3.65
XP_001651187.1	protease m1 zinc metalloprotease [*Aedes aegypti*]	2724452	−2.37
ADV40172.1	cysteine protease [*Latrodectus hesperus*]	2064675	−1.79
EGI57993.1	sentrin-specific protease 7 [*Acromyrmex echinatior*]	1812196	1.62
ACK57788.1	cathepsin C [*Litopenaeus vannamei*]	1961192	1.75
EFN69039.1	gamma-glutamyltranspeptidase 1 [*Camponotus floridanus*]	2724894	1.87
NP_001026086.1	probable carboxypeptidase PM20D1 precursor [*Gallus gallus*]	445655	2.46
XP_001843240.1	matrix metalloprotease [*Culex quinquefasciatus*]	2733315	2.77
XP_972146.1	matrix metalloprotease [*Tribolium castaneum*]	564795	3.03
proPO cascade			
AAV91026.1	serpin-6 [*Manduca sexta*]	2717112	−1.88
CAA72032.2	masquerade-like protein [*Pacifastacus leniusculus*]	2334552	−1.74
ACR43430.1	serine proteinase inhibitor [*Macrobrachium rosenbergii*]	2727642	1.52
ACY66494.3	serine protease [*Scylla paramamosain*]	1687763	2.05
XP_967003.1	serine protease, partial [*Tribolium castaneum*]	797270	2.4
EFA11957.1	serine protease H82 [*Tribolium castaneum*]	2731772	2.55
ACA28963.1	melanization inhibition protein [*Pacifastacus leniusculus*]	1606934	2.83
ACC77765.1	taicatoxin serine protease inhibitor precursor [*Oxyuranus scutellatus*]	1030751	3.33
Antioxidant enzymes			
ZP_01905434.1	oxidoreductase, short chain dehydrogenase [*Plesiocystis pacifica* SIR-1]	301453	−1.65
XP_003701560.1	glutathione S-transferase C-terminal domain-containing protein [*Megachile rotundata*]	1291687	1.53
XP_002126758.1	peroxiredoxin 3 [*Ciona intestinalis*]	597640	1.78
XP_003769788.1	glutathione S-transferase Mu 5-like [*Sarcophilus harrisii*]	249723	1.82
Coagulation			
XP_971239.1	hemolectin CG7002-PA [*Tribolium castaneum*]	1258851	1.53
ABI95361.1	hemolymph clottable protein [*Litopenaeus vannamei*]	2564133	1.93
ABW77320.1	clottable protein 2 [*Penaeus monodon*]	2723153	1.99
ABK59925.1	clottable protein [*Marsupenaeus japonicus*]	181026	1.99
ABK60045.2	alpha-2-macroglobulin [*Litopenaeus vannamei*]	329710	2.02

Table 4 (*continued*)

Accession No.	Gene name	Contig ID	Fold change
Others			
ACX68556.1	chitinase 6 [*Litopenaeus vannamei*]	2714206	−2.8
AEI88100.1	ubiquitin conjugating enzyme 7 interacting protein-like protein[*Scylla paramamosain*]	563041	1.79
EHJ76300.1	ubiquitin-conjugating enzyme E2 J2 [*Danaus plexippus*]	1212195	1.87
XP_001662806.1	ubiquitin conjugating enzyme 7 interacting protein [*Aedes aegypti*]	2682580	1.92

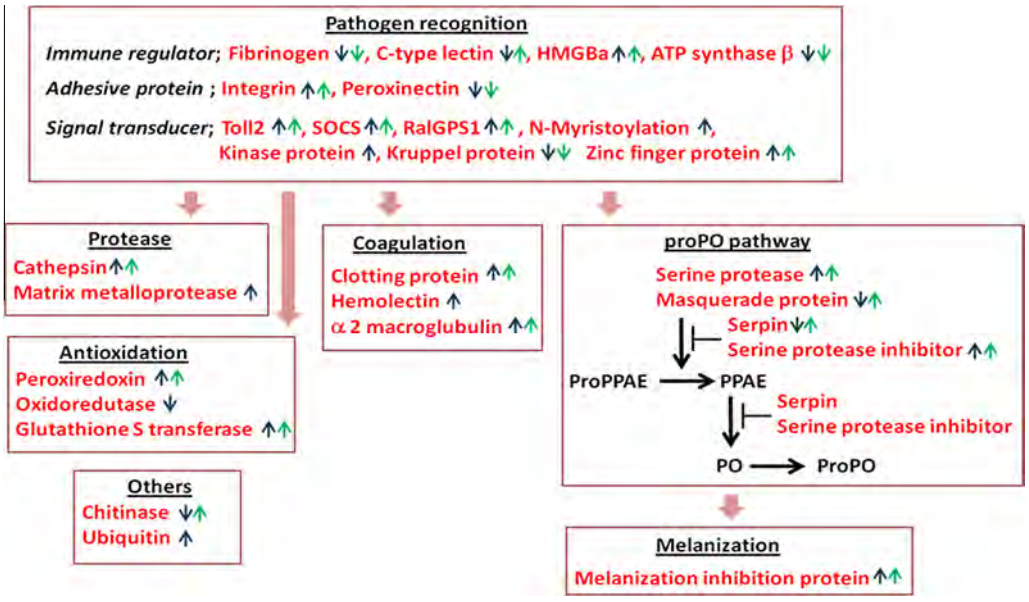

Fig. 2. Putative pathway of immune response functions associated with resistance to TSV in *L. vannamei* based on RNA-Seq transcriptome analysis. The blue and green arrows referred to significant up and down regulation in RNA-Seq transcriptome analysis and QPCR results, respectively. HMGBa = high mobility group box a, SOCS = suppressors of cytokine signaling, RalGPS1 = ras-specific guanine nucleotide-releasing factor 1.

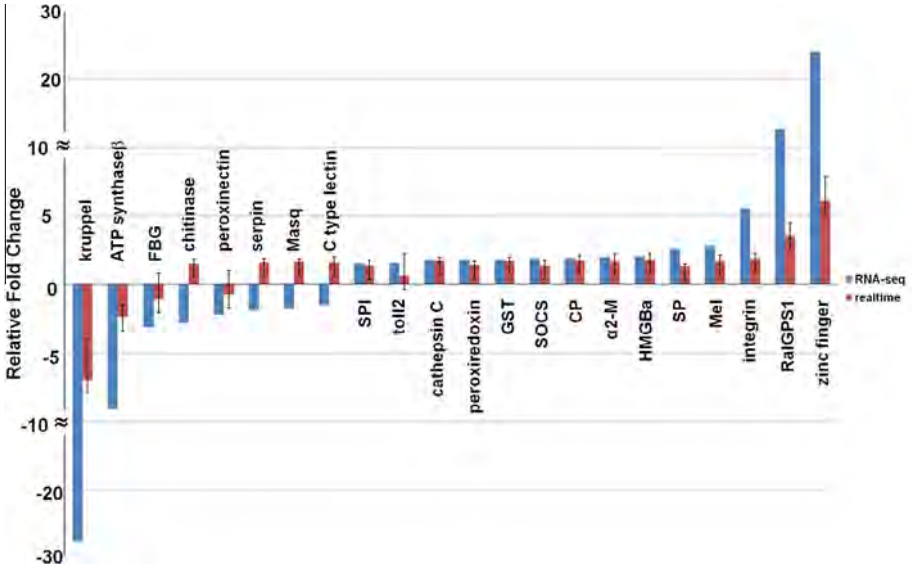

Fig. 3. Comparison of relative fold change of RNA-Seq and QPCR results between the TSV-resistant and susceptible *L.vannamei* hemocyte. The transcript expression levels of the selected genes were each normalized to that of the β-actin gene. FBG = fibrinogen, Masq = masquerade, SPI = serine protease inhibitor, GST = glutathione-s-transferases, SOCS = suppressors of cytokine signaling, CP = clottable protein, α2-M = α2-macroglobulin, HMGBa = high mobility group box a, SP = serine protease, Mel = melanization interactin protein and RalGPS1 = ras-specific guanine nucleotide-releasing factor 1.

immune pathways could be clearly observed in the transcriptome anlaysis of hemocyte while those involve in growth and energy metabolism are more pronounced in hepatopancreas transcriptome.

In this work, a total of 697 unigenes were identified to be differentially expressed and associated with TSV resistance. These differentially expressed genes were assigned multiple potential functions in molecular function and biological process (Supplementary Table 4). Here, we mainly focused on the immune-related genes enriched and mediated the TSV resistance. Taking the advantages of GO annotation and manual imputation of putative function via literature search of studies in several model organisms, we categorized these significantly differentially expressed genes into the following categories, including pathogen/antigen recognition, coagulation, proPO cascade, antioxidation, and protease (Fig. 2, Table 4). As more biological knowledge and genomic data become publicly available, such methodological developments will lead to a better understanding on the mechanism of disease resistance in shrimp. Below we highlight key constituents of these categories and their potential functions in the context of host immune responses to TSV resistance.

4.1. Pathogen recognition

Innate immune responses in crustacean have been induced by minute quantities of pathogen associated molecular patterns (PAMPs) (Janeway and Medzhitov, 1999) such as bacterial lipopolysaccharides and peptidoglycans as well as fungal β-1,3-glucans. Several organisms have many ways to recognize as well as eliminate pathogen including immune regulator, adhesive protein activity and signal transduction (Akira et al., 2006).

Of our prime interest, TSV resistance resulted in severe transcriptional suppression of genes involved in Kruppel-like factors (KLFs). Kruppel homolog 1 isoform A and kruppel-like protein 1 were highly down-regulated around 20-fold in the resistant strain in contrast to most other differentially expressed immune genes which were up-regulated or showed smaller decreases in expression (Table 4). This down-regulation in the TSV resistant strain as compared with susceptible strain, attracted our attention and led us to hypothesize the interaction between KLFs and shrimp TSV resistance. It has been reported that KLFs contains a family of transcription factors that belongs to Sp1-like proteins which contain three highly conserved Cys2–His2 zinc-finger DNA-binding domains in the C-terminal region (Kaczynski et al., 2003; Chang et al., 2012). They can bind to GC-rich sequences, such as the GC- and CACCC-boxes in promoters. Functionally, KLFs have been shown to be involved in various functions, including hematopoiesis, differentiation, the development of lymphocytes, determination of stem cells and programmed cell death (Bieker, 2001; Kaczynski et al., 2003; Pearson et al., 2008). The expression pattern of KLFs here in our study is consistent with that in shrimp WSSV infection, in which *Pm*KLF was considered to be important to WSSV infection. Suppression of the expression of *Pm*KLF in white spot syndrome virus (WSSV) infected-shrimp caused delay of cumulative mortalities, suggesting that *Pm*KLF have crucial roles in transcription and viral infection (Chang et al., 2012). Follow-up studies to examine KLF expression in different shrimp families, strains, and species with differing TSV resistance and susceptibility are needed to confirm the roles of KLF in resistance against viral diseases.

In contrast to the situation of KLF, strong induction of RAS-specific guanine nucleotide-releasing factor (RalGPS) was captured in the RNA-Seq results. RalGPS belongs to the Ras-related protein superfamily (Valencia et al., 1991; Feig, 1994; Quilliam et al., 2002). The Ras family of small GTPases contains over 60 small (20–25 kDa) guanine-nucleotide binding proteins that are involved in the control of a variety of cellular processes, such as phagocytosis, vesicle transportation, cellular differentiation, proliferation and apoptosis (Bar-Sagi and Feramisco, 1985). Several different signaling mechanisms can be induced to activate Ras such as tyrosine kinases, G-protein-coupled receptors, adhesion molecules, second messengers, and various protein-interaction modules to relocate and/or activate guanine nucleotide exchange factors (GEFs) and elevate intracellular Ras-GTP levels. In crustacean, transcript expression of Ras family proteins such as Rab and Ran in *Marsupenaeus japonicus* were reported to be up-regulated after WSSV challenge and involved in counteracting virus infection (Han and Zhang, 2007; Zong et al., 2008; Zhao et al., 2011). Interestingly, similar pattern of RalGPS was apparent in the expression profile in TSV-resistant line, likely indicating its common expression pattern of immune responses in shrimp disease resistance.

In addition, genes such as fibrinogens (FBG), calcium-dependent (C-type) lectins, peroxinectin, and serine/threonine-protein kinases were slightly induced. Being responsible for carbohydrate-binding and pathogen-binding, FBG in *M. japonicus* has been reported to be potentially involved in immune defense against several bacterial and viral pathogens by recognizing and binding to cell surface (Chai et al., 2012). C-type lectins, containing a carbohydrate recognition domain (CRD), play important roles in innate immunity, cell–cell interactions, and especially pathogen recognition. In marine invertebrates, C-type lectins have been identified in scallop, as well as in shrimp (Luo et al., 2006; Liu et al., 2007; Ma et al., 2007; Yang et al., 2007; Sun et al., 2008; Zhang et al., 2009). The C-type lectins identified in shrimp have been reported to be associated with defense against bacterial and viral pathogens as pattern-recognition receptors (PRRs) (Junkunlo et al., 2012; Wei et al., 2012). As a cell adhesion protein, peroxinectin was first isolated and purified from the freshwater crayfish *Pacifastacus leniusculus* and demonstrated to have activities of cell adhesion (Johansson and Söderhäll, 1988; Liu et al., 2004). It is released in response to a stimulus and activated outside of cells to mediate hemocyte attachment and spread (Johansson and Söderhäll, 1988; Johansson et al., 1999; Liu et al., 2005). The mechanism of peroxinectin in crustacean cellular defense reaction was presented as the strong adhesion to the foreign target forming a multilayered sheath of cells during encapsulation (Johansson, 1999). The emerging patterns may indicate a relative conservation expression profile of the pathogen recognition in shrimp. However, further profiling in additional shrimp tissues is still needed for a more comprehensive perspective.

More intriguingly, several zinc-finger proteins were shown modest fold of changes including shn zinc finger protein, zinc finger protein 628, zinc finger protein 585B, zinc finger protein FYVE domain-containing protein, and zinc finger protein 658B-like. A zinc finger and BTB domain-containing protein 37-like isoform 1 was by far among the most highly up-regulated gene observed in our differentially expressed set, with expression increasing 27.5-fold. Interestingly, zinc-finger proteins are mainly recognized in DNA-binding proteins including transcription factors (Mocchegiani et al., 2005; Large and Mathies, 2010). Previous studies reported that zinc-finger antiviral protein (ZAP) is a host factor that inhibits the replication of several virus pathogens by preventing the accumulation of viral mRNA in the cytoplasm (Chen and Varani, 2005). During recent years, the differentially expressed genes in hemocytes of the crayfish (*Procambarus clarkii*) infected with WSSV were studied by using suppression subtractive hybridization (SSH) and cDNA microarrays. The results reported that the transcript level of zinc-finger protein gene was significantly up-regulated after infection. Thus, zinc-finger protein may be involved in immune defense responding to the virus infection in various organisms (Sera, 2009; Zeng and Lu, 2009; Zimmerman et al., 2009; Jeong et al., 2010). Our results of the expression pattern of the zinc finger

proteins are consistent with what was found in the above studies, which potentially indicated that zinc finger proteins probably play a critical role in shrimp TSV-resistance events in *L. vannamei*.

4.2. Prophenoloxidase (proPO) cascade

In our results, an array of components of the prophenoloxidase (proPO)-activating system were perturbed such as serine proteases, masquerade, serine protease inhibitors, serpin as well as melanization inhibition protein. While the overall pattern was suggestive of stimulation in the TSV-resistant line, there were notable exceptions with suppression pattern.

The proPO-activation pathway, consisting of pattern recognition proteins, serine proteases, and their inhibitors, is an important component of the innate immune response of insects. Several studies reported that proPO system has been involved in wound healing and melanotic encapsulation (Sritunyalucksana et al., 1999; Sritunyalucksana and Söderhäll, 2000; Li and Xiang, 2013). ProPO is an inactive form and is converted to an active form phenoloxidase (PO) after limited proteolysis by serine proteinases (Cerenius and Söderhäll, 2004). Then, PO catalyzes the early steps in the pathway to melanin formation. The proPO-activating roles in the immune defense reactions was reported in *P. leniusculus* (Söderhäll and Cerenius, 1998), *P. californiensis* (Vargas-Albores et al., 1993a,b) and *P. monodon* (Sritunyalucksana et al., 1999). In addition, ProPO was first cloned from the freshwater crayfish *P. leniusculus* in 1995 (Aspán et al., 1995). In *L. vannamei*, the expression of proPO transcript was demonstrated that different proPOs have different expression profiles; and the sites for proPO synthesis vary among different arthropod species (Wang et al., 2006b). Recently, proPO-b was observed that expression was down-regulated in *L. vannamei* challenged with white spot syndrome virus (WSSV) suggesting that proPO-b is a main transcript form of proPO gene in *L. vannamei*, and it may play a role in defence against WSSV virus. (Ai et al., 2008). Considering the shrimp prophenoloxidase system (Amparyup et al., 2013), here, we provided more evidence of the critical roles of the proPO system in the shrimp immune responses against major pathogens. Apparently, the roles of proPO system in disease resistance need to be given greater attention.

4.3. Others

Chitinase was among a smaller set of immune genes induced to differential expression. Having been found to be required for several mechanisms such as morphogenesis of cell walls and exoskeletons in most of vertebrate and invertebrate, the chitinase participates in several biological mechanisms, such as nutrient digestion, morphogenesis, pathogenesis and pathogen defense (Spindler-Barth et al., 1990; Kono et al., 1995; Wang et al., 2002; Mali et al., 2004; Dahiya et al., 2006; Duo-Chuan, 2006; Zhu et al., 2008). Chitinase was previously reported to be up-regulated in the hepatopancreas of WSSV-resistant *L. vannamei* (Huang et al., 2010; Proespraiwong et al., 2010). In our study, chitinase 6 was seen significantly induced as well. Taken together, chitinase may probably play an important role in acting against the viral pathogen infection.

5. Conclusions

Transcriptomic analysis of hemocyte from TSV-resistant and susceptible *L. vannamei* lines were first successfully constructed and several differentially expressed genes were identified. A total of 697 unigenes were significantly altered associated with TSV resistance. We observed dysregulation in a number of genes involved in diverse pathways including pathogen/antigen recognition, coagulation, proPO pathway cascade, antioxidation, and protease. A growing understanding of shrimp immune responses to viral pathogens should lead to the identification of shared and pathogen/tissue specific signatures with utility as disease biomarkers and/or targets for improved vaccination strategies to the shrimp aquaculture.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2013.07.020.

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