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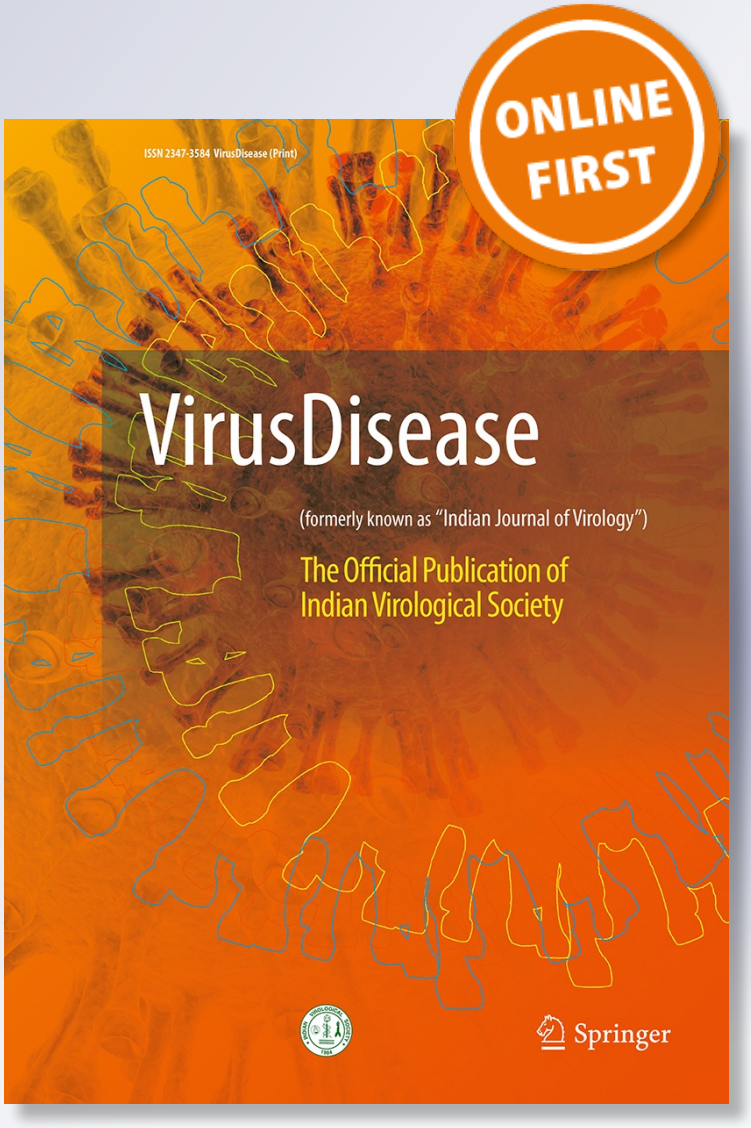

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ORIGINAL ARTICLE

Gene expression profiling in gill tissues of *White spot syndrome virus* infected black tiger shrimp *Penaeus monodon* by DNA microarray

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Abstract *White spot syndrome virus* (WSSV) continues to be the most devastating viral pathogen infecting penaeid shrimp the world over. The genome of WSSV has been deciphered and characterized from three geographical isolates and significant progress has been made in developing various molecular diagnostic methods to detect the virus. However, the information on host immune gene response to WSSV pathogenesis is limited. Microarray analysis was carried out as an approach to analyse the gene expression in black tiger shrimp *Penaeus monodon* in response to WSSV infection. Gill tissues collected from the WSSV infected shrimp at 6, 24, 48 h and moribund stage were analysed for differential gene expression. Shrimp cDNAs of 40,059 unique sequences were considered for designing the microarray chip. The Cy3-labeled cRNA derived from healthy and WSSV-infected shrimp was subjected to hybridization with all the DNA spots in the microarray which revealed 8,633 and 11,147 as up- and down-regulated genes respectively at different time intervals post infection. The altered expression of these numerous genes represented diverse functions such as immune response, osmoregulation, apoptosis, nucleic acid binding, energy and metabolism, signal transduction, stress response and molting. The changes in gene expression profiles observed by microarray analysis provides molecular insights and framework of genes which are up- and down-regulated at different time intervals during WSSV infection in shrimp. The microarray data was validated by Real Time analysis of four differentially expressed genes involved in apoptosis (translationally controlled tumor protein, inhibitor of apoptosis protein, ubiquitin conjugated enzyme E2 and caspase) for gene expression levels. The role of apoptosis related genes in WSSV infected shrimp is discussed herein.

Keywords Microarray · WSSV · *Penaeus monodon*

Introduction

DNA microarray, one of the high-throughput and effective strategies for analyzing gene transcription profiles has been used for studying WSSV pathogenesis and transcriptional analysis of immune genes in response to WSSV infection. The use of microarray technique has led to the identification of genes with common or similar roles in the WSSV infection cycle which could be grouped in the same hierarchical cluster [1] or genes which were expressed at early and late stages of WSSV infection [2]. Several other studies have reported the successful use of microarray in detecting WSSV genes transcribed at different stages of WSSV infection such as immediate-early genes [3, 4], early genes at 6 hpi (hours post infection) [5], genes at 2, 6, 12 and 24 hpi [6] and latency related genes [7].

At the host level, in one of the first low-density cDNA microarray based experiments containing 100 genes, the altered expression of various cellular genes involved in immunity such as lipopolysaccharide, β-1,3 glucan binding protein, serine protease, C-type lectin, macrophage mannose receptor, low density lipoprotein receptor along with

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structural and genes of unknown functions were obtained from the hepatopancreas of WSSV infected *Penaeus stylirostris*) [8]. Wang et al. [9] analysed gene expression patterns in the cephalothorax of *Fenneropenaeus chinensis* at 6 h and at moribund stage of WSSV infection. The low-density cDNA microarray with 3,136 spots revealed altered expressions of chaperones, calcium-dependent genes and genes involved in cell structure and energy metabolism. A DNA microarray chip consisting of 1,026 genes selected from 4 different EST libraries was used for identification of differentially expressed immune genes which revealed highest number of up- and down-regulated genes at 24 hpi in haemocytes of WSSV-challenged *Penaeus monodon* [10]. Robalino et al. [11] generated a cDNA microarray encompassing 2,469 putative unigenes expressed in gills, circulating haemocytes and hepatopancreas of *Litopenaeus vannamei.* This microarray chip was used for analyzing the transcriptomic response of shrimp to WSSV infection which revealed up-regulation of genes encoding antimicrobial effectors and down- regulation of genes involved in protection against oxidative stress. The sequences of 9,990 cDNAs obtained from *P. monodon* EST database were used in developing a microarray chip for analysing transcriptional changes in haemocytes of *P. monodon* in response to viral (WSSV, yellow head virus) and bacterial (*Vibrio harveyi*) pathogens. The highest number of differentially expressed genes were found in shrimp infected with WSSV [12].

In the present study, shrimp cDNAs were printed onto a microarray chip based on 40,059 unique sequences to identify differentially expressed genes in response to WSSV infection. A total number of 19,780 differentially expressed genes unravelled by microarray analysis were further characterized for gene ontology distribution and comparative analysis of gene expression profiles. The gene expression analysis of the four apoptosis related genes (translationally controlled tumor protein (TCTP), inhibitor of apoptosis protein (IAP), ubiquitin conjugated enzyme E2 and caspase) was carried out in this study to decipher their functional role and in understanding the molecular mechanisms by which they interact and involve in the apoptosis network that occur in shrimp in response to WSSV infection.

Materials and methods

Shrimp

Black tiger shrimp *P. monodon,* were collected from culture farms located at Chennai, India. The shrimp (average body weight 30 g) were distributed in 13 FRP tanks of 500 L capacity for the WSSV challenge experiment and were acclimatized in aerated seawater of 28 ppt salinity. The water quality parameters were recorded and monitored daily. The pH and temperature of the seawater ranged from 7.6 to 7.9 and 28 to 30 °C respectively. The shrimp were fed pelleted feed throughout the experimental period.

WSSV challenge experiment

WSSV viral stock was prepared using infected *P. monodon* tissue which was minced, centrifuged and the resultant supernatant filtered. After confirming the presence of WSSV by PCR, the viral stock solution was subjected to tenfold dilution. The virus stock was estimated to contain 2.62×10^6 μL^{-1} viral copies by Real Time analysis. A total of 96 shrimp were used for the challenge experiment. Four group of 18 shrimp each were challenged with 0.1 mL of 10^{-7} virus dilution of virus stock via the intramuscular route. The experiment was carried out in triplicate for all time points with 6 shrimp in each tank. Gill tissues were collected from the WSSV infected shrimp at 6, 24, 48 h and at moribund stage (post 48 h up to 72 h) post WSSV infection. One group (24 shrimp) was maintained as a control throughout the experiment in a single tank. The tissue samples were stored in RNA*later* solution (Qiagen, Germany) for further analysis.

Microarray-chip preparation

All sequences used from NCBI database were of *P.monodon* only. A total of 42,013 sequences which included 39,397 of ESTs, 1,854 of genes and 762 gene sequences generated earlier in our laboratory from cDNA clones were considered for designing a custom made 8 × 60 K Agilent chip (Genotypic Technology Pvt Ltd, Bangalore, India). The EST sequences available in the NCBI database were derived from cDNA libraries constructed from various shrimp tissues. Probes of length 60 mer with an average of one probe per target sequence were designed as per the standard algorithms and methodologies used by Agilent technologies for 40,059 unique sequences after removing redundant sequences. Probe selection and distribution was done based on target specificity which was validated through local alignment using BLAST tool. Agilent's 8 × 60 K custom designed array format contained 61,657 experimental and 1,319 control features. Based on standard phosphoramadite chemistry, the in situ synthesized oligonucleotide microarray in which the oligos are synthesized directly on the glass microarray surface was used in the experiment. Spotting on Agilent's 8 × 60 K custom designed microarray was carried out using Agilent SurePrint technology. The blanks were filled with random replicates of duplicated probes used in the same array. Two

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slides of 8 × 60 K array format were used in the experiment.

RNA extraction and RNA quality control

Six pooled gill tissues of WSSV infected shrimp in triplicate were collected at each of the different time intervals post infection. Total RNA from pooled gill tissues was extracted using QIAzol method as per the manufacturer's instructions (Qiagen, Germany). The concentration and purity of the RNA extracted were evaluated using the Nanodrop Spectrophotometer (Thermo Scientific: 1000). The integrity of the input template RNA was determined prior to labeling/amplification and hybridization using the Agilent 2100 bioanalyzer. The RNA was considered to be of good quality based on the 260/280 values, rRNA 28S/18S ratios and RNA integrity number (RIN) obtained by Bioanalyzer. For each time point the total RNA extracted from six pooled gill tissues collected from un-infected (-control group) shrimp served as control for the microarray experiment.

RNA labelling, amplification and hybridization

Cy3-labeled complementary RNA was generated using Quick-Amp labeling Kit (Agilent) following T7 promoter based-linear amplification method (One-Color Microarray-Based Gene Expression Analysis, Agilent) following the manufacturer's instructions. Briefly, total RNA (500 ng) was reverse transcribed using oligodT primer tagged to T7 promoter sequence. The cDNA thus obtained was converted to double stranded cDNA in the same reaction. Further, the cDNA was converted to cRNA in the in vitro transcription step using T7 RNA polymerase enzyme and Cy3 dye was added into the reaction mix for incorporation during cRNA synthesis. The cRNA obtained was purified using Qiagen RNeasy columns (Qiagen, Germany). Concentration and amount of dye incorporated was determined using Nanodrop. The Cy3-labeled cRNA (600 ng) was subjected to hybridization with all the DNA spots in the microarray using gene expression hybridzation kit (Agilent) in hybridization chamber at 65 °C for 16 h. Hybridized slides were washed using Agilent gene expression wash buffers. Two groups of samples were set for hybridization, each containing experimental (WSSV) infected shrimp collected at different time periods (6, 24, 48 h and moribund stage) and the uninfected control group. Hybridization was carried out on sixteen samples which included three biological repeats for each of the infected samples collected at different time points. The cRNA extracted from six uninfected samples was used as control for each time interval. The experimental design and the raw data for each hybridization was submitted to GEO data repository which supports MIAME compliant data submission (http://www.ncbi.nlm.nih.gov/geo).

Microarray data analysis

The hybridized and washed microarray slides were scanned on a G2505C scanner (Agilent) and the images were quantified using feature extraction software (version-10.7, Agilent). The feature extracted raw images were analyzed with GeneSpring GX Version 12 software (Agilent). Percentile shift normalization (percentile shift normalization is a global normalization, where the locations of all the spot intensities in an array are adjusted) was done in GeneSpring GX using the 75th percentile shift. Differential gene expression with significant up- regulation of genes were identified using the cutoff fold >0.6 (log base2) and <− 0.6-fold (log base2) for down-regulation of genes respectively in three replicate of test samples with respective uninfected control samples. Statistical *T* test and p-value were calculated based on Student's *T* test algorithm. Differentially regulated genes were clustered using hierarchical clustering based on Pearson coefficient correlation algorithm to identify significant gene expression patterns. Genes were classified based on their functional analysis which was performed based on the EST sequences of the designed probes and subjected to BLAST X. The BLAST hits showing >50 % alignment length were considered for the uniprot annotations.

Gene expression analysis by Real Time PCR

Total RNA was extracted using NucleoSpin RNA II kit (Macherey–Nagel, Germany) from six pooled gill tissues of control and infected shrimp at different time intervals post infection. Total RNA was converted to cDNA using Protoscript M-MuLV first strand cDNA synthesis kit (New England Biolabs, USA). The cDNA was used to analyze the relative expression of the selected four differentially expressed apoptosis related genes (TCTP, IAP, ubiquitin conjugated enzyme E2 and caspase) identified from microarray analysis. Real Time PCR was carried out using Power SYBR green PCR master mix (Applied Biosystems, USA). Real Time primers were designed with the aid of Primer Express software (Applied Biosystems, USA). The gene specific primers used for Real Time analysis are shown in Table 1. The shrimp β-actin gene was amplified with primers (β-actin-F and β-actin-R) based on GenBank accession no. JN808449 which was used as an endogenous control. This sequence of β-actin gene has been previously used to design gene-specific primers. Gene expression levels of immune genes of *P. monodon* by Real Time PCR has been analysed using β-actin as an internal control [13]. The relative quantification of the transcripts were assessed

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Table 1 Gene specific primers used for Real Time analysis

Gene	Real Time primer sequence (5′–3′)	Expected PCR product size (bp)	Gen Bank accession no.
Translationally controlled tumor protein (TCTP)	F: TCAGCTGAAGAGGCAGATGAAG	100	JX961663
	R: TCTTGACTTGGAAGCCGGTTT		
Ubiquitin conjugated enzyme E2	F: CAGCTGATCCCCTCGTAGGA	80	JX961664
	R: CCAGAGCCGTGCAATTCTATC		
Inhibitor of apoptosis protein (IAP)	F: ACCCAGAAGCCAAGTGATTTA	110	EF114675
	R: TCATCTTTCTCCCAGTTACGA		
Caspase	F: GCCGAGAACCTCAGGAAT	101	DQ846887
	R: TCGGATTCGTAGAGGACTGC		
β-actin	F: CCCTGTTCCAGCCCTCATT	90	JN808449
	R: GGATGTCCACGTCGCACTT		

by comparative ΔC_T method and the results were expressed as the fold change in levels of the gene expression. The fold change for the expression of specific gene target was calculated from the Ct values using the standard formula: Fold change $= 2^{(-\Delta\Delta Ct)}$ (stepone software V2.1 Applied Biosystems, USA). The statistical analysis of the data for comparison between groups was carried out by one-way ANOVA and the values with $p < 0.05$ were considered significant.

Results

Gene expression analysis by microarray

The microarray revealed a total number of 27,556 genes that were differentially up- or down- regulated in three replicate samples analysed at each different time intervals of 6, 24, 48 h and moribund stage of infection. A set of common genes that were differentially expressed in all the three replicates were identified which were either up- or down- regulated. A total number 2,114, 2,884, 1,105 and 2,530 were obtained as up-regulated common genes and 3,035, 3,542, 1,830 and 2,740 were found to be common down-regulated genes amongst the three replicates at 6, 24, 48 h and moribund stage of infection respectively. These differentially expressed genes (19,780) were considered in further analysis for gene ontology distribution and comparative gene expression profiles.

Gene ontology (GO) annotation

Gene Ontology distribution of the differentially expressed common genes identified at different time intervals was carried out for the three major categories of biological process, cellular component and molecular function (Supplementary Table 1). A total number of 3,126 genes could be distributed in these three categories based on gene ontology. The biological process category which represented the genes belonging to cellular and metabolic process, biological regulation and biological adhesion revealed highest number of up-regulated genes (168) and down-regulated genes (162) at 24 and 48 hpi respectively. The cellular component category represented by genes belonging to cell structure, organelles and components showed the highest number of up-regulated genes (201) and down-regulated genes (165) at 24 hpi respectively. In the molecular function category, the genes involved in molecular binding and catalytic activity revealed the highest number of up-regulated genes (173) and down-regulated genes (260) at 24 and 6 hpi respectively. Interestingly, 24 hpi revealed the highest number of both up-regulated genes (542) and down-regulated genes (484) for all the three GO categories.

Comparative analysis of gene expression profiles

From these differentially expressed genes, a total of 54 genes representing different functional categories were selected for comparative analysis of gene expression profiles. These genes represented wide functional categories such as cytoskeletal proteins, cell cycle, protein synthesis and processing, immune response, osmoregulation, apoptosis, RNA binding and processing, DNA binding, energy and metabolism, signal transduction, stress response, virus receptor proteins and molting.

At 6 hpi 16 genes were up-regulated and the highest gene expression was observed for nucleoside diphosphate kinase (2.94-fold). Amongst 38 down-regulated genes, β-1,3-glucan-binding protein was observed to be the most down-regulated gene with −6.05-fold change. At 24 hpi, 20 genes were up-regulated and the highest gene expression was observed for ribonuclease (2.96-fold). Lysozyme was observed to be the most down-regulated gene with

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−2.46-fold change amongst 34 down-regulated genes. At 48 hpi 34 genes were up-regulated and the highest gene expression was observed for lysozyme (2.1-fold). Penaeidin was observed to be the most down-regulated gene with −2.77-fold change amongst 20 down-regulated genes. At moribund stage of infection 38 genes were up-regulated and the highest gene expression was observed for myosin (2.91-fold). Amongst 16 down-regulated genes, penaeidin was observed to be the most down-regulated gene with −4.7-fold. The gene expression levels with fold changes are shown in Supplementary Table 2. The differentially expressed transcripts were grouped based on gene expression profile similarity by hierarchical clustering which resulted in three expression patterns of up-regulated, repressed and late induced transcripts (Fig. 1).

Gene expression analysis by Real Time PCR

The gene expression profiles obtained by Real Time PCR for the four genes involved in apoptosis (TCTP, IAP, ubiquitin conjugated enzyme E2 and caspase) is shown in Fig. 2. Real Time PCR revealed down-regulation of TCTP gene from early infection stage at 6 h (0.336-fold), 24 h (0.197-fold), 48 h (0.126-fold) to moribund stage of infection (0.072-fold). The IAP gene revealed down-regulation at 6 h (0.26-fold), 24 h (0.96-fold) and up-regulation at 48 h (1.49-fold) and moribund stage of infection (1.63-fold). The caspase gene showed down-regulation at all time points post infection from 6 h (0.103-fold), 24 h (0.069-fold), 48 h (0.179-fold) to moribund stage of infection (0.256-fold). Ubiquitin conjugated enzyme E2 gene showed up-regulation at all time points post infection from 6 h (1.13-fold), 24 h (1.53-fold), 48 h (1.64-fold) to moribund stage of infection (2.04-fold).

Discussion

The shrimp ESTs provide information on the host differential expression at the transcriptional level at a given experimental condition. In the absence of the shrimp whole genome sequence, shrimp ESTs are useful for gene identification and probe preparation for cDNA microarrays. In the present study we have used 40,059 unique sequences of *P. monodon* to develop the microarray chip, which resulted in identification of a total number of 27,556 differentially expressed genes. Amongst the three replicates, 8,633 (31 %) and 11,147 (40 %) common genes were identified which were either up- or down-regulated at different time intervals of post infection respectively.

We selected gill tissues of *P. monodon* to analyse differentially expressed genes in response to WSSV infection as the gills which is of ectodermal and mesodermal tissue of origin is the primary site for viral replication and the prevalence of virus is reported to be high in this tissue [14]. Identification and altered expression profiles of immune genes have been reported using the gill tissues of WSSV infected penaeid shrimp [15, 16].

The hierarchical clustering of the differentially expressed transcripts based on the similarity in gene expression profiles, showed cluster I of genes (ribophorin, calnexin, ubiquitin-conjugating enzyme, Na^+/K^+-ATPase, ribonuclease, dopamine receptor and HSP 90) to be up-regulated at all the four time points post infection. In case of heat shock proteins (HSPs) similar results have been reported in other studies in which the HSPs (HSP70, HSP90) were observed to up-regulated in response to WSSV infection in shrimp [9]. The seasonal variation in WSSV prevalence has been observed to occur in shrimp farms with high incidence of disease outbreaks during the cold season [17]. The HSP70 is reported to get induced at higher temperature in WSSV infected shrimp and specific dsRNA silencing of HSP70 led to increased susceptibility of shrimp to WSSV infection [18]. These studies indicate the functional role of shrimp HSPs which can be attributed to the temperature associated protection observed in shrimp against WSSV.

The cluster II of genes (TCTP, caspase, QM protein, penaeidin, arginine kinase, phosphoenolpyruvate carboxykinase, receptor for activated protein kinase, 5-HT1 receptor and lymphoid organ expressed YHV receptor) were found to be down-regulated at all the four time points post infection. Penaeidins, which are generally known to possess antibacterial and antifungal activities are reported to play a potential role in antiviral immunity in shrimp infected with WSSV [19]. The host gene response patterns of antimicrobial molecule penaeidin observed in this study was in agreement to the findings of Wongpanya et al. [10], who reported the transcripts of penaeidin to be down-regulated after 24 hpi in haemocytes of WSSV-challenged *P. monodon*. In another study, the expression of penaeidin (PEN5) transcripts in the haemocytes of WSSV-challenged *P. monodon* by Real Time PCR was shown to be down-regulated after WSSV-challenge except at 24 hpi when 1.8-fold increase in the expression level was observed [19]. As several class of penaeidins have been isolated from different shrimp species, it would be interesting to identify the class of penaeidins exhibiting antiviral response against WSSV.

The down-regulation of genes that are involved in energy metabolism such as arginine kinase suggests that the shrimp metabolism is affected by WSSV infection [9]. In a recent study, arginine kinase in *L. vannamei*, (*Lv*AK) was shown to interact with VP14 of WSSV and to be involved in WSSV infection [20]. In another important finding, the shrimp arginine kinase was suggested to be a component of

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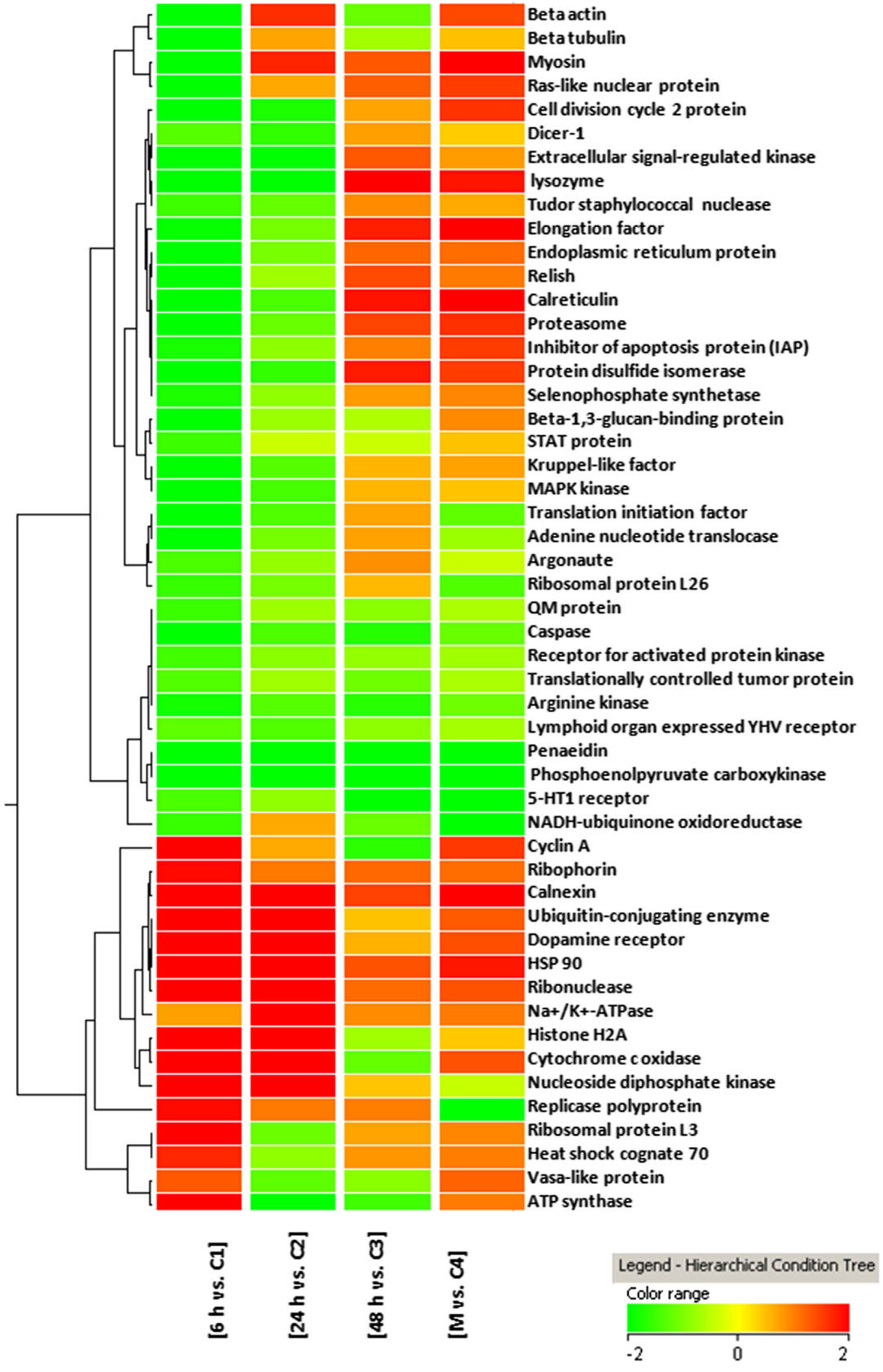

Fig. 1 Hierarchical clustering of differentially expressed transcripts based on gene expression profile similarity

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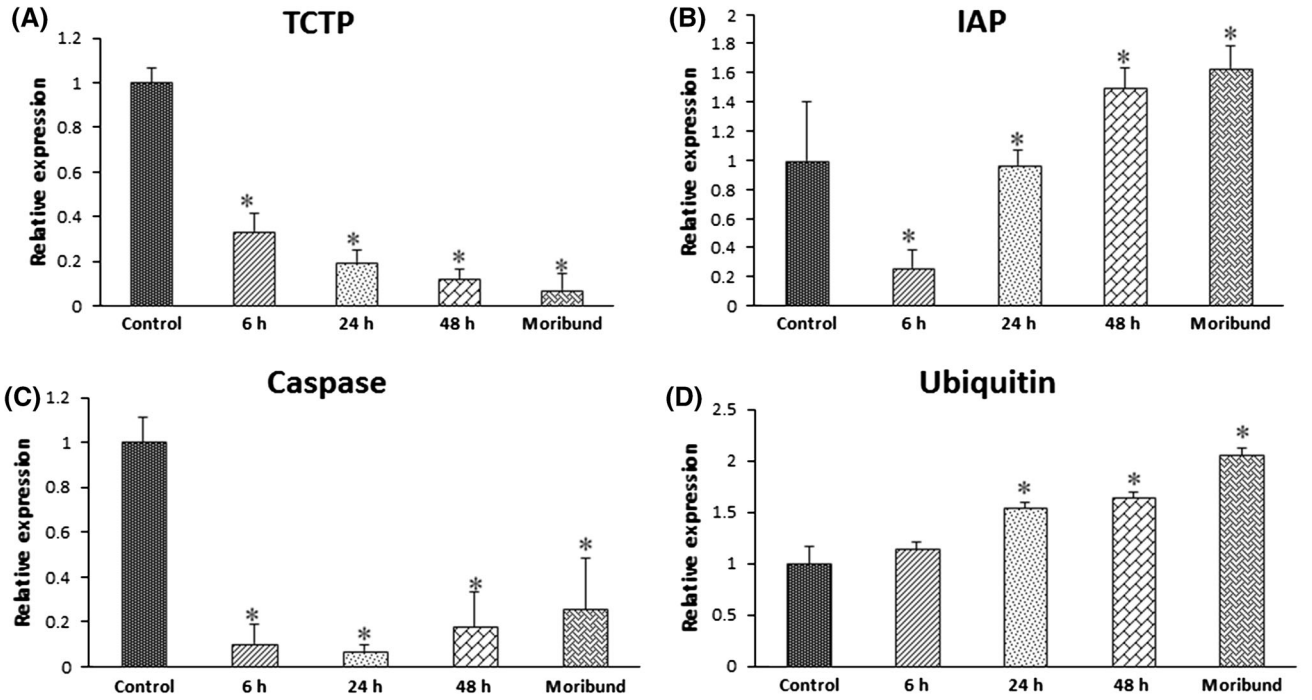

Fig. 2 Gene expression analysis of apoptosis related genes in WSSV infected gill tissues of *Penaeus monodon* at 6, 24, 48 h and moribund stage of post infection by Real Time PCR. **a** Translationally controlled tumor protein (TCTP) **b** Inhibitor of apoptosis protein (IAP) **c** caspase **d** ubiquitin conjugated enzyme E2. The significant difference ($p < 0.05$) in gene expression levels is indicated with *asterisks*

RNA inducing silencing complex. The direct interaction of *Marsupenaeus japonicus* arginine kinase with VP28 siRNA indicated that shrimp arginine kinase plays a crucial role in RNAi mechanism to protect shrimp against WSSV infection [21].

The late induced genes which constituted the third category included cell division cycle 2 protein, dicer-1, extracellular signal-regulated kinase, lysozyme, tudor staphylococcal nuclease, elongation factor, endoplasmic reticulum protein, relish, calreticulin, proteasome, IAP, protein disulfide isomerise, selenophosphate synthetase, β-1,3-glucan-binding protein, signal transducer and activator of transcription (STAT), kruppel-like factor and MAPK kinase. This cluster represented genes, the expression levels of which were higher after 24 or 48 h post infection. In the present study, it is interesting to observe that STAT was one of the genes which was up-regulated at the late stage of infection. In shrimp STAT is reported to get annexed by WSSV to enhance the expression of immediate early genes [22].

Apoptosis is an intriguing phenomenon in WSSV infected shrimp. Both virus and shrimp use apoptosis and anti-apoptosis related genes to elicit respective responses for enhancing viral replication or as a defence mechanism against WSSV infection. We, therefore selected TCTP, IAP and caspase the three apoptosis related genes and in addition ubiquitin conjugated enzyme E2 gene for gene expression analysis in WSSV infected shrimp by Real Time PCR.

TCTP is an important shrimp protein which has been suggested to have a potential role in subverting viral infection by preventing apoptosis induced by WSSV. TCTP is well conserved in both animal and plant kingdom and plays an important role in cellular process. This gene has been renamed as fortilin in a study related to human TCTP in which it was shown to be involved in cell survival as anti-apoptotic protein [23]. In the present study, by microarray analysis we observed TCTP to be down-regulated at all time points post infection. The gene expression levels obtained in microarray were comparable with those obtained by Real Time PCR which revealed down-regulation of TCTP gene from early infection stage at 6 h to moribund stage of infection. The down-regulation of TCTP during WSSV infection indicates that apoptosis may be a cause of shrimp mortality associated with the infection. As a result of down-regulation of TCTP gene expression, the virus infected shrimp cells cannot survive. This is in agreement with other studies in shrimp, in which the role of TCTP as anti-apoptotic protein has been suggested. Bangrak et al. [24] reported decrease in TCTP expression levels in haemocytes of severely symptomatic shrimp infected with WSSV as compared to asymptomatic but WSSV-infected

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shrimp. This indicates that TCTP plays a role in protecting shrimp from viral induced mortality. A recombinant TCTP protein was shown to provide protection which resulted in 80–100 % survival of shrimp after infection with WSSV probably by inhibiting viral replication [25]. In another study, Graidist et al. [26] reported up-regulation of TCTP in shrimp haemolymph during the early phase of WSSV infection with abrupt decrease in expression in moribund stage of the shrimp. WSSV-infected shrimp were found to have fivefold greater expression of TCTP than normal shrimp. It is suggested that TCTP helps in cell protection against apoptosis by blocking etoposide-induced caspase 3 activation. The reduced expression levels of TCTP from early stage to moribund stage of infection observed in this study, supports the hypothesis that shrimp mortality is associated with apoptosis which is induced by WSSV [27].

In a recent study, the overexpression of *Pm*-fortilin in Sf9 cells was observed to prevent the expression of WSSV associated early and late genes such WSSV-DNA polymerase, VP15 and VP28 but not an immediate early gene ie1. This indicates that fortilin may have a functional role during early and late stages of WSSV infection by reducing the expression of WSSV genes [28]. The silencing of TCTP by dsRNA led to increased viral loads in WSSV infection in *L. vannamei*, indicating that TCTP was involved in shrimp immune responses against WSSV infection [29]. The TCTP is suggested to bind to the GDP/GTP free form of Rab proteins as the structural studies of the TCTP from *Schizosaccharomyces pombe*, revealed that TCTPs form a structural superfamily with the Mss4/Dss4 family of proteins [30]. The Rab GTPase is shown to be involved in the phagocytic immune response against WSSV in *M. japonicus* through a protein complex of beta-actin, tropomyosin, and VP466 of WSSV [31]. It is therefore, speculated that TCTP interacts with Rab GTase to induce phagocytosis against the virus [29].

Inhibitors of apoptosis proteins (IAPs) which are found in insects to humans are involved in suppressing apoptosis. In shrimp, IAPs have been isolated from *L. vannamei* and *P. monodon*. IAP from *P. monodon* (*Pm*IAP) was shown to block apoptosis induced by IAP antagonist *Drosophila* Rpr in insect cells. It was further demonstrated that there was physical interaction between *Pm*IAP and *Drosophila* Rpr and both co-localized in same cellular locations. The baculoviral IAP repeat (BIR) domains BIR2 and BIR3 of *Pm*IAP were found to be involved in inhibiting apoptosis induced by Rpr suggesting *Pm*IAP use mechanisms similar to *Drosophila* and mammals to regulate apoptosis [32]. In *L. vannamei* three IAP genes from (*Lv*IAP1, *Lv*IAP2 and *Lv*Survivin) have been characterized. *Lv*IAP1, an orthologue of *Pm*IAP was suggested to play major role in regulation of shrimp haemocyte apoptosis and was found essential for shrimp survival [33]. IAPs isolated from *L. vannamei* were shown to be up-regulated post infection suggesting protective roles of IAPs in shrimp defense against WSSV infection [34]. In the present study, by microarray analysis we observed IAP to be down-regulated at initial stages (6 and 24 h) of WSSV infection and subsequently up-regulated from 48 hpi. The gene expression levels obtained in microarray are in concordance with the results obtained by Real Time PCR which revealed down-regulation of IAP gene at early infection stage at 6 and 24 h and up-regulation at 48 h and moribund stage of infection. The down-regulation of IAP gene observed in the present study at early stages of WSSV infection might have promoted apoptosis to facilitate the spread of virus particles to other adjacent cells. As a result of accelerated WSSV infection, the up-regulation of IAP at 48 h and moribund stage of infection may be ineffective in blocking the virus induced apoptosis. However, further work needs to be carried out to comprehend the mechanism underlying this hypothesis.

The presence of a zinc-binding baculoviral IAP repeat (BIR) domain is a characteristic feature of members of the IAP family. The BIR domains which act as the protein–protein recognition domains are required for caspase binding [35]. A full length *Pm*IAP of 4,769 bp with an ORF encoding a protein of 698 amino acids is reported to contain three BIR domains and a RING domain [32]. The partial gene sequence obtained for IAP in this study (Gen Bank accession no. KJ768439) also revealed the three BIR domains. Hence, we selected caspase gene in addition to TCTP and IAP for gene expression analysis to investigate the role of this gene in apoptosis.

The caspases, which belong to cysteine proteases and known to execute apoptosis have been identified from *F. merguiensis* [36], *P. monodon* [37, 38], *L. vannamei* [39, 40] and *M. japonicus* [41]. Caspases are classified into two categories, the initiator caspases and the effector caspases which are characterized by an extended N-terminal prodomain (>90 amino acids) or 20–30 residues in its prodomain sequence respectively [42]. The full-length caspase gene isolated in this study with a 954 bp open reading frame, encoding 317 amino acid (GenBank accession no. KJ768440) was similar to the earlier reported caspase gene from *P. monodon* (DQ846887). The caspase gene displayed the characteristic caspase domains with a potential conserved active site (QACRG pentapeptide) suggesting that it belonged to effector class of caspases as reported by others [38, 40]. The shrimp caspases play a potential role in the host defense against WSSV infection. In WSSV-challenged *M. japonicus* shrimp survivors, the caspase gene was observed to be significantly up-regulated and the caspase gene silencing by RNAi assays resulted in inhibition of WSSV-induced apoptosis [41]. The dsRNA knockdown of *L. vannamei* caspase which led to significant

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protection against mortality from a low-dose WSSV challenge [39] and acceleration in WSSV replication [40] suggested protective role of caspase in shrimp against WSSV infection. In the present study, the microarray analysis revealed caspase to be down-regulated at all time points post infection. The gene expression levels obtained in microarray were similar to the results obtained by Real Time PCR which showed down-regulation of caspase gene from 6 h to moribund stage infection. Interestingly, as mentioned above we observed up-regulation of IAP gene expression in later stages (48 h and moribund stage) of infection. This increase in gene expression of IAP might be responsible for inhibition of caspase activity by IAP. One of the mechanisms suggested in regulating apoptosis by IAPs is by inhibiting caspases directly [43]. The down-regulation of caspase gene also indicates the role of WSSV induced apoptosis resulting in mortality of infected shrimp. Our experiment indicates that apoptosis results in increased shrimp mortality after viral challenge which may be due to virus-induced apoptosis of shrimp. Viruses are reported to have developed several strategies to interfere with the proteolytic activity of caspases. For example, the ORF390 of WSSV is reported to encode anti-apoptotic protein and is involved in apoptosis regulation by inhibiting the caspase activity [44]. This results in interference of apoptosis thereby aiding in prolonging the life of the infected cell with increased viral replication and persistence [45]. Interestingly, a recent study has identified the role of WSSV miRNA (WSSVmiR-N24) in suppressing of host antiviral apoptosis by downregulating the caspase 8 expression in *M. japonicus* indicating that WSSV-miR-N24 target the host caspase 8 and regulates apoptosis in shrimp by inhibiting the host antiviral immune response thereby facilitating viral replication [46]. These studies suggests that similarly, the caspase in *P. monodon* may be required for defending against WSSV infection.

Some IAPs are reported to posess (RING) finger domain which functions as an E3 ubiquitin ligase, thereby recruiting E2 ubiquitin-conjugating enzymes and transfer ubiquitin to target proteins which bind to IAPs such as caspase. Ubiquitination may therefore catalyze the proteasome-mediated degradation of these target proteins [35]. Ubiquitin conjugated enzyme E2 gene in the present study, showed up-regulation at all time points post infection from 6 h to moribund stage of infection, indicating that it might have a functional role in antiviral response by ubiquitinating WSSV. Ubiquitin-conjugating enzyme E2 isolated from *Fenneropenaeus chinensis* was shown to inhibit WSSV replication and ubiquitinate WSSV RING domain-containing proteins [47]. However, the role of ubiquitin conjugated enzyme E2 in apoptosis remains to be established and requires further investigation.

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