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Analysis of Gene Expression in Haemocytes of Shrimp *Penaeus monodon* Challenged with White Spot Syndrome Virus by cDNA Microarray

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ABSTRACT: A cDNA microarray technique was employed to identify differentially-expressed genes in the black tiger shrimp (*Penaeus monodon*) challenged with white spot syndrome virus (WSSV). The cDNA chip was composed of 718 unique genes from *P. monodon* and 308 unique genes from the kuruma shrimp (*Marsupenaeus japonicus*). The cDNA arrays were hybridized with Cy3/Cy5-labeled probes generated from the haemocyte RNA of *P. monodon* injected with WSSV. The gene expression profile was determined at different time points after injection. The results showed differential expression in haemocytes of WSSV-challenged *P. monodon*. The number of up- and down-regulated genes was highest at 24 h after WSSV injection. The pathogen invasion resulted in up-regulation of ribosomal proteins, indicating an increase in protein synthesis. The expressions of several immune-related genes also changed. The mRNA expressions of three highly responsive genes (calmodulin, tubulin and asialoglycoprotein receptor) in response to shrimp pathogens, WSSV and the luminescence bacteria *Vibrio harveyi*, were verified by real-time PCR and confirmed the involvement of these genes in shrimp defense. Our results suggested the WSSV infection altered the expression of a wide range of cellular genes. The microarray analysis identified several clones with unknown function that were up-regulated by WSSV infection. The roles of these clones in shrimp immune response need to be further investigated.

KEYWORDS: microarray, shrimp, real-time PCR, gene expression,WSSV.

INTRODUCTION

Shrimp farming has continued to expand and become a major source of income in many countries. The biggest obstacle to the future of the shrimp industry is outbreaks of infectious diseases[1]. White spot syndrome virus (WSSV) is a major pathogen in shrimp that causes high mortality and huge economic losses in shrimp aquaculture[2]. WSSV virion has been isolated and identified as a double-stranded circular DNA virus. Its genome containing 305 kilo-bases was sequenced[3]. Although considerable progress has been made in the characterization of WSSV, the understanding of shrimp defense system in response to the viral infection is still poor. Knowledge of the immune system in shrimp is necessary to develop methods to control and minimize the loss of production due to infectious diseases.

In crustaceans, the innate immune system has cellular and humoral components, and is localized mainly in haemolymph which contains different types of haemocytes[4]. Several proteins and peptides are synthesized and stored in haemocytes and released into the haemolymph upon infections[5]. Van de Braak *et al.*[6] studied the clearance of *Vibrio* in the shrimp *Penaeus monodon* and showed that haemocytes play important roles in bacterial clearance. Several immune molecules were identified from shrimp haemocytes by means of molecular approaches. Expressed sequence tag analysis has been used to identify immune-related genes in haemocytes and the hepatopancreas of two sister species of *Litopenaeid* shrimps: Pacific white shrimp (*Litopenaeus vannamei*) and Atlantic white shrimp (*L. setiferus*)[7]. A similar approach has also been successfully used for discovery of immune genes in haemocytes of shrimps *Marsupenaeus japonicus*[8], *P. monodon*[9] and *Fenneropenaeus chinensis*[10]. Nevertheless, only a small number of immune genes have been characterized. Larger numbers of immune-related genes need to be identified and functionally characterized to better understand shrimp immunity.

Several techniques have been implemented in gene expression analysis i.e. differential display PCR (DD-PCR), suppression subtractive hybridization (SSH) and microarray analysis. In shrimp, DD-PCR, a technique for analyzing differences in gene expression, was used to determine changes in gene expression patterns in hemocytes of *P. monodon* upon *V. harveyi* infection[11]. Recently, Pan *et al.*[12] employed SSH, a sensitive PCR-base subtraction approach, to identify differentially expressed genes in virus-resistant penaeid shrimp. However, microarray analysis is fast becoming the method of choice for the detection of gene expression on the grand scale.

cDNA microarray analysis is used to analyze changes in expression of thousands of genes at the same time. This technology is also used for detection of mutations in specific genes and measurement of gene expression in diseased versus healthy organisms. The approach provides an attractive solution for effectively identifying candidate genes involved in the pathogenesis of diseases. This technique could generate new hypotheses for the mechanisms underlying the development of damage and might enhance the understanding in pathogenesis of diseases in the penaeid shrimp. Dhar *et al.*[13] have used cDNAs microarray to compare gene expression patterns in the hepatopancreas of healthy and WSSV-infected shrimp (*Penaeus stylirostris*). This approach was also used to study viral gene expressed in WSSV-infected and specific-pathogen free shrimp[14].

In the present study, we used cDNA microarray to examine changes in gene expression of the black tiger shrimp *P. monodon* challenged with WSSV injection. The expressions of three genes, calmodulin, tubulin and asialoglycoprotein receptor, were found to be strongly up-regulated by the viral infection, both by the microarray analysis and by quantitative real-time PCR, suggesting the involvement of these genes in the pathogenesis of WSSV.

Materials and Methods

Shrimp Samples

Specific pathogen-free shrimp (30-35 g) *P. monodon* were obtained from a Domestication Program in Nakhonsrithammarat province (Thailand) and divided into 4 groups. The first and the second groups were injected with WSSV and lobster haemolymph medium (LHM). The third and the fourth groups were injected with *V. harveyi* and 0.85% (w/v) NaCl, respectively. The second and the fourth groups were control shrimp. All groups were acclimatized in aquaria at ambient temperature (28 ± 4 °C) and salinity of 15 ppt for at least 1 day before used in the experiments.

Pathogens

WSSV stock solution was obtained from the Charoenpokaphand Group of Companies, Thailand. The viral copy number was determined by real-time PCR. The initial viral stock containing 8.8×10^6 copies of WSSV was diluted 100 fold with LHM. *V. harveyi* strain 1526 was cultured in Tryptic Soy Broth (TSB, 2% NaCl) at 30 °C for 8 to 10 hours (h) and diluted 1:1000 with 0.85% NaCl. Cell density was determined by the plate count method[15].

Infection and Haemolymph Collection

For the pathogen challenge, 100 µl containing either 7.6×10^5 viral copies of WSSV or 10^5 cfu of *V. harveyi* were intramuscularly injected into the fourth abdominal segment of the shrimp.

At different time points after the injection, haemolymph was collected from the ventral sinus in the first abdominal segment using a 24G× 1 inch needle fitted onto a 1-ml syringe. Each syringe was pre-filled with 200 µl of anti-coagulant (10% (w/v) sodium citrate). The haemolymph was collected from *P. monodon* at 4 time points after the WSSV or LHM injection (6, 24, 48 and 72 hour post injection (hpi)) and at 3 time points after the *V. harveyi* or 0.85% NaCl injection (6, 24, and 48 hpi). In the latter case, most of the animal died after 48 h. The injected animals were checked for WSSV infection by PCR method[16]. *V. harveyi* infection was checked by culturing the suspensions of hepatopancreas of injected shrimp on TSA plates supplemented with 2% (w/v) NaCl and incubating at 30 °C overnight. Colonies of *V. harveyi* 1526 from infected shrimp were identified as the strong luminescent spots in the dark.

Total RNA Isolation

Total RNA was extracted from the haemocytes of the pathogen-challenged shrimp and the control shrimp injected at the indicated time points using TRIzol (Invitrogen Life Technologies, USA) according to the manufacturer's protocol. Equal amount of the total RNA from 10 individuals at each time point was pooled.

Gene Expression Profiling Using Microarray Analysis

The cDNA microarray chip was prepared from cDNA clones of two penaeid shrimps *P. monodon* and *M. japonicus*. A total of 1,026 genes spotted on the array were selected from 4 different EST libraries: two are haemocyte cDNA libraries of *V. harveyi* injected- and non-injected *P.monodon* (718 clones) whereas the other two are haemocyte cDNA libraries of WSSV injected- and non-injected *M. japonicus* (308 clones). These cDNA clones represent known genes in different functional

categories as well as unknown ESTs. The cDNA clones were amplified by PCR according to the standard protocol. The primers were designed to anneal the vector regions (sense 5'-GTGCTGCAAGGCGATTAAGTTGG-3', antisense 5'-TCCGGCTCGTATGTTGTGTGGA-3'). The PCR products were purified and concentrated using a 96 PCR cleanup kit (Millipore, USA) to attain a final concentration at least 500 μg/ml. The printed slides were processed by DNA Chip Research Inc. (Tokyo, Japan). Each gene was spotted on slide in duplication. Probes were prepared from the challenged and the control shrimp and used to hybridize on the imprinted glass slides as follows. The total RNA (25 mg) was used to synthesize first strand cDNAs labeled with aminoallyl-dUTP simultaneously using a LabelStar Array Kit (QIAGEN, Germany) according to the manufacturer's instructions. The cDNAs were purified using a QIAquick PCR Purification Kit (QIAGEN, Germany) following the manufacturer's protocol. The purified cDNAs were further labeled with Cy3 (control shrimp injected with LHM) and Cy5 (WSSV challenged shrimp). The labeled probes were mixed and denatured at 95 °C for 2 minutes before adding the 100 nM oligo $d(T)_{18}$ which is used for blocking poly(A)$^+$ sequences within the cDNA arrayed on the glass slide. Hybridization was then carried out for 16 h at 42°C, followed by several washing and rinsing steps (washing with 2× SSC-0.1% SDS for 20 min at room temperature, 0.2× SSC-0.1% SDS for 20 min at room temperature, two washes of 0.2×SSC-0.1% SDS for 20 min at 55 °C, 0.2× SSC-0.1% SDS for 20 min at room temperature with gentle shaking and two rinses with 0.2× SSC and 0.05× SSC at room temperature). The glass slides were dried and scanned immediately using a GenePix 4000B Microarrays Scanner (Amersham Biosciences, USA). The scanned images were analyzed with the GenePix Pro 3.0 program (Axon Instrument, Union City, CA). After subtracting the background fluorescence, differences in the Cy5 and Cy3 incorporation efficiencies were corrected by global normalization. The results were expressed as the gene expression ratio, i.e., the ratio of the intensities of Cy5:Cy3. The signal intensity was calculated from the intensity means of duplet spots and was subtracted from the background signal. Genes with feature ratios over 2.0 and under 0.5 were considered as up-regulated and down-regulated, respectively. The data management and expression analysis was performed according to the MIAME (Minimum Information About a Microarray Experiment) checklist[17]. The complete data set has been submitted to and accepted by ArrayExpress, the microarray database of the European Bioinformatics Institute[18]. The accession number is A-MEXP-306.

The processed data were further analyzed using the Gene Cluster 3 developed by Michael Eisen[19] for cluster analysis. Tree View software available on the Stanford site was also used to generate visual representations of the classification. Hierarchical clustering classifies samples according to their overall gene expression profiles and group genes on the basis of correlations of their expression level pattern in all samples[20, 21].

Verification of Gene Expression Using Real Time RT-PCR

The relative expressions of 3 genes with significantly altered expression, calmodulin, asialoglycoprotein receptor and tubulin genes, were further determined by real time RT-PCR (SYBR Green), using alpha-1 elongation factor (EF-1α) as a reference gene. The specific primers for these genes were designed based on the sequences of their cDNA clones, shown in Table 1.

Total RNA was extracted from the WSSV or *V. harveyi*-challenged shrimp and the control shrimp as described above, and was treated with DNaseI. One microgram of pooled total RNA (10 individuals at each time point) was reverse transcribed with oligo (dT). cDNA was synthesized with an ImProm-II Reverse Transcription System (Promega, USA) according to the manufacturer's instructions. The targeted cDNAs were amplified in a reaction volume of 25 μl containing IQ SYBR Green Supermix (Bio-Rad) with an iCycler IQ

Table 1. List of primers used for the detection of calmodulin, asialoglycoprotein receptor, tubulin and EF-1a gene by SYBR Green Real-time RT-PCR.

Gene	Primer	Primer sequence (5'-3')	%GC	Amplicon size (bp)
calmodulin	CaM-F	CAGTTCCTTGGTGGTGAT	50	131
	CaM-R	CGATTGGCTTGTGATACA	44	
asialoglycoprotein receptor	ASGPR-F	GAACGAGAATGCTGACCTGA	50	232
	ASGPR-R	AGTATTTGCGAGTATGGGAG	45	
tubulin	Tubulin-F	GAAAACACCGATGAAACTTACTG	39	239
	Tubulin-R	GAGAGGAGCAAAACCAGGCAT	52	
EF-1a	EF-F	GGTGCTGGACAAGCTGAAGGC	61	148
	EF-R	CGTTCCGGTGATCATGTTCTTGAT	45	

Real-time Detection System (Bio-Rad, Hercules, CA). The specific primers were used at a final concentration of 0.2 μM with 5μl (1:10) cDNA template. The real-time PCR protocol consisted of 8 min at 95°C followed by 40 cycles of 10 s at 95°C, 15 s at 55°C and 10 s at 72°C. For each sample, the amplification plot and corresponding dissociation curves were examined. All samples were run in triplicate. In each 96-well plate, a standard curve was generated from a serial dilution of the cDNA of the target gene and was used to determine of the mRNA copy number of samples. For each time point, the amount of the target genes and a reference gene (EF-1α) was determined. Then, the target amount was normalized by the reference amount to obtain relative expression values. Statistical significance was determined via a one way ANOVA analysis ($p<0.05$).

The relative expression of the respective gene was also normalized to a sample from shrimp injected with saline solution or LHM. Correlation coefficients for the standard curves were > 0.996 for all genes. The relative expression ratio (R) of the gene was calculated using the following equation[22]:

$$R = \frac{\left(E_{target}\right)^{\Delta Ct_{target}\left(control-sample\right)}}{\left(E_{ref}\right)^{\Delta Ct_{ref}\left(control-sample\right)}}$$

Results and Discussion

The shrimp cDNA microarrays used in this study consisting of 1,026 distinct genes in duplicate spotting: 718 genes from *P. monodon* and 308 genes from *M. japonicus*. The functions of 489 of these genes were unknown. The full data set can be accessed at http://www.ebi.ac.uk/arrayexpress/ with the accession number A-MEXP-306. After shrimp injected with WSSV, the highest number of up-regulated genes was observed at 72 hpi whereas a large number of down-regulated genes were found at 24 hpi (Table 2). The genes strongly up- and down-regulated by WSSV injections was shown in Table 3. Only a small number of *M. japonicus* genes spotted on the array cross-hybridized with the labeled cDNA from *P. monodon*.

To explore the relationship among the expressed genes in the haemocytes of the WSSV- challenged shrimp, the microarray data were processed by Gene Cluster 3 analysis. The data was filtered and further examined by hierarchical clustering analysis. Hierarchical clustering is a pairwise average linkage analysis of gene expression data. A tree represents the relationships among genes whose branch lengths reflect the degree of similarity between the genes. The hierarchical clustering and dendrogram of WSSV-injected shrimp haemocytes at four different time points

Table 2. Numbers of up- and down-regulated genes* in haemocytes of the black tiger shrimp after injection with WSSV.

Expression of genes		Number of Genes			
		6 hpi	24 hpi	48 hpi	72 hpi
Up	Known genes	19	28	32	38
	Unknown genes	9	35	33	44
	Total	28	63	65	82
Down	Known genes	1	13	18	10
	Unknown genes	1	59	40	37
	Total	2	72	58	47
	Total	**30**	**135**	**123**	**129**
		(2.92%)	(13.16%)	(11.99%)	(12.57%)

*The cDNA chip is composed of 1,026 unique genes including 489 unknown genes.
hpi: hour post injection.

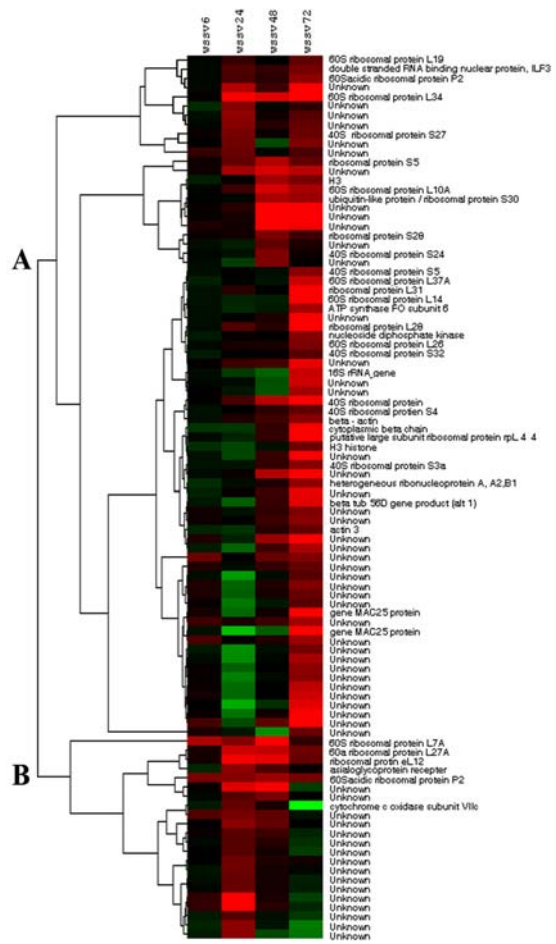

Fig 1. Hierarchical clustering analysis of 96 genes at 4 time points (6, 24, 48 and 72 hpi) in haemocytes of WSSV-challenged shrimp. Each row represents a single gene and each column an experimental sample. Genes were linked by the dendrogram shown on the left to illustrate similarity in their expression pattern. The brackets on the left represent the identified clusters, namely, group A and group B. Up-regulated genes are red and down-regulated genes are green.

Table 3. List of known genes showing high response to white spot syndrome virus challenge at different time points.

Accession Number	Description	Fold changes[a]
WSSV challenge at 6 hpi		
Up regulated genes		
CO576813	40S ribosomal protein S24	10.35
DW042577	asialoglycoprotein recepter	7.12
DW042596	cellular nucleic acid binding protein	5.05
BI018085	tubulin beta-1 chain	4.70
AU175611	60S ribosomal protein L27	4.62
CO408860	60S ribosomal protein L7A	4.30
AU175516	elongation factor 2	4.24
DW042540	von Hippel-Lindau binding protein 1	3.67
AU175729	40S ribosomal protein S9	3.43
BI784454	cytosolic manganese superoxide dismutase percursor	3.41
DW042744	Beta actin	3.17
CO576814	40S ribosomal protein S25	3.06
AU176130	60S ribosomal protein L38	2.98
CO576842	60S acidic ribosomal protein P2	2.78
CO576795	40S ribosomal protein S10	2.61
CO576806	60S acidic ribosomal protein P2	2.59
CO408870	RpS25 gene product	2.55
AU176244	40S ribosomal protein S19	2.30
EE332498	60S ribosomal protein L6	2.22
Down regulated genes		
DW042635	kupffer cell receptor	0.43
WSSV challenge at 24 hpi		
up regulated genes		
AU176186	60S ribosomal protein L34	24.10
CO408866	60S ribosomal protein L30	21.66
AU176065	14-3-3-like protein	19.33
AU176183	Rat insulinoma gene-Rig	8.92
DW042577	asialoglycoprotein recepter	8.72
CO576787	60S ribosomal protein L12	7.51
AU175456	calmodulin	7.20
CO576786	60S ribosomal protein L13	6.16
CO408860	60S ribosomal protein L7A	5.80
CO576778	60S ribosomal protein L27A	5.37
CO408905	proteasome subunit beta type 2	4.49
AU176216	QM protein	3.84
DW042609	chain H, cytochrome Bc1 complex	3.81
AU176245	calcium-sensitive chloride channel 2	3.27
DW042739	ATP synthase alpha chain, mitochondrial precursor	3.24
CO576842	60S acidic ribosomal protein P2	3.02
CO408860	60S ribosomal protein L7A	2.90
AU176135	40S ribosomal protein S3	2.68
AU175758	40S ribosomal protein S27	2.65
CO576805	40S ribosomal protein S18	2.52
AU176256	integral membrance protein 2A	2.39
CO576795	40S ribosomal protein S10	2.37
DW042690	cytochrome c oxidase subunit VIIc	2.24
CO576817	40S ribosomal protein S5	2.19
AU176046	60S ribosomal protein L19	2.16
EE332498	60S ribosomal protein L6	2.10
CO576770	60S ribosomal protein L28	2.07
DW042705	double stranded RNA binding nuclear protein	2.03
down regulated genes		
DW042746	hypothetical protein	0.49
DW042578	beta tub 56D gene product (alt 1)	0.47
EE332504	gene MAC25 protein	0.42
AU175457	CG6848 gene product	0.38
DW042717	actin related protein	0.34
AU176107	hemocyte protein-glutamine gamma-glutamyltransferase	0.34

Table 3. Cont'd.

Accession Number	Description	Fold changes[a]
BI018085	tubulin alpha-1 chain	0.26
CO408863	60S ribosomal protein L17	0.21
EE332500	probable reverse transcriptase	0.13
BI784441	Penaeidin-2 precusor	0.07
DW042535	agrin precursor	0.07
DW042977	penaeidin-3k	0.06
BI784443	11.5 kDa antibacterial protein	0.06
WSSV challenge at 48 hpi		
up regulated genes		
DW042631	translocon-associated protein	24.33
AU175456	calmodulin	22.90
BI018085	tubulin beta-1 chain	15.54
CO408860	60S ribosomal protein L7A	7.85
CO576787	60S ribosomal protein L12	6.70
EE332503	arginine kinase	6.41
AU176186	60S ribosomal protein L34	6.15
CO408861	60S ribosomal protein L10A	5.85
CO576778	60S ribosomal protein L27A	4.90
AU175496	myosin regulatory light chain	4.87
CO576805	40S ribosomal protein S18	4.81
AU176195	40S ribosomal protein	4.56
AU175516	elongation factor 2	4.45
DW042526	alpha-2-tubulin	4.24
CO576817	40S ribosomal protein S5	4.23
CO408854	ubiquitin-like protein / ribosomal protein S30	3.97
DW042538	actin 1	3.86
EE332502	H3	3.66
CO576842	60S acidic ribosomal protein P2	3.65
CO576786	60S ribosomal protein L13	3.33
CO576808	60S ribosomal protein L24	3.22
AU176256	integral membrance protein 2A	3.04
CO408857	40S ribosomal protein S24	2.83
CO576784	40S ribosomal protein S28	2.73
AU175323	alpha-tubulin	2.47
DW042749	cytoplasmic A3	2.40
EE332498	60S ribosomal protein L6	2.33
AU176107	hemocyte protein-glutamine gamma-glutamyltransferase	2.32
DW042652	acyl coenzyme A dehydrogenase, long chain	2.29
CO576782	40S ribosomal protein S7	2.21
AU176065	14-3-3-like protein	2.17
AU176216	QM protein	2.11
down regulated genes		
AU175755	mitochondrial 1rRNA gene, partial 3' end	0.48
AU175571	mitochondrial rRNA gene	0.48
AU175717	16S rRNA gene	0.47
EE332504	gene MAC25 protein	0.46
AU175955	partial mitochondrial 16S rRNA gene	0.46
AU175931	mitochondrial, complete genome	0.46
EE332500	probable reverse transcriptase	0.46
AU175787	16S rRNA gene	0.45
AU175567	profilin	0.44
AU175739	16S rRNA gene	0.43
AU175654	40S ribosomal protein S	0.42
AU175405	16S ribosomal RNA gene	0.41
DW042717	actin related protein	0.31
DW042535	agrin precursor	0.23
BI784443	11.5 kDa antibacterial protein	0.12
BI784441	Penaeidin-2 precusor	0.10
DW042977	penaeidin-3k	0.10
AU175296	cytochrome c oxidase subunit 2	0.06

Table 3. Cont'd.

Accession Number	Description	Fold changes[a]
WSSV challenge at 72 hpi		
up regulated genes		
AU175456	calmodulin	20.78
CO408865	60S ribosomal protein L14	18.57
AU176186	60S ribosomal protein L34	17.51
CO408869	putative large subunit ribosomal protein rpL 44	17.35
AU175697	60S ribosomal protein L7A	15.35
EE332504	gene MAC25 protein	15.20
BI784458	heat shock cognate 70 kDa protein	13.78
CO576771	60S ribosomal protein L31	12.42
DW042511	cytoplasmic beta chain	12.37
CO576770	60S ribosomal protein L28	12.08
AU176195	40S ribosomal protein	11.01
DW042578	beta tub 56D gene product (alt 1)	6.50
EE332499	hypothetical protein F08F1.8	6.44
AU175780	60S ribosomal protein L37A	5.34
AU175787	16S rRNA gene	5.08
CO408861	60S ribosomal protein L10A	3.96
DW042938	ATP synthase FO subunit 6	3.74
CO408854	ubiquitin-like protein	3.52
CO576842	60S acidic ribosomal protein P2	3.27
AU175239	heterogeneous ribonucleoprotein A, A2,B1	3.20
DW042744	beta – actin	3.10
CO408855	5S ribosomal protein	3.00
CO408886	40S ribosomal protein S3a	2.99
DW042845	nucleoside diphosphate kinase	2.96
CO576795	40S ribosomal protein S10	2.86
CO408859	60S ribosomal protein -like	2.72
EE332502	H3	2.66
CO576842	60S acidic ribosomal protein P2	2.60
DW042705	double stranded RNA binding nuclear protein, ILF3	2.59
CO576778	60S ribosomal protein L27A	2.44
EE332498	60S ribosomal protein L6	2.43
AU175758	40S ribosomal protein S27	2.42
CO576787	60S ribosomal protein L12	2.27
DW042665	actin 3	2.24
DW042564	60S ribosomal protein L19	2.22
CO408853	40S ribosomal protien S4	2.22
CO576817	40S ribosomal protein S5	2.21
CO408851	40S ribosomal protein S32	2.04
down regulated genes		
EE332501	histone 1	0.49
AU175798	16S rRNA gene	0.49
CO408897	eukaryotic translation initiation factor 3, subunit 2	0.47
AU175755	mitochondrial 1rRNA gene, partial 3' end	0.40
AU175745	NADH ubiquinone oxidoreductase chain 6	0.33
BI784443	11.5 kDa antibacterial protein	0.27
AU175457	CG6848 gene product	0.14
DW042690	cytochrome c oxidase subunit VIIc	0.13
BI784441	Penaeidin-2 precusor	0.10
DW042977	penaeidin-3k	0.10

[a] Genes with mean of ratio over 2.0 and under 0.5 were considered as up- and down- regulated genes, respectively.

were shown in Fig 1. Ninety-six genes were filtered and grouped into two clusters according to the following expression patterns. Group A, consisting of 74 genes, was characterized by elevated expression at a late time (72 hpi). Twenty-two genes of the group B cluster were classified by an increase in expression at 24 to 48 hpi. Group A genes, with increased expression at 72 hpi, mainly encode ribosomal proteins and several unknown genes. Increased expression of transcripts for ribosomal proteins have also been observed in fish challenged with bacteria[23], human challenged with bacteria[24] and shrimp challenged with WSSV [7, 12].

For group B, some ribosomal proteins showed transient expression at 24 and 48 hpi. Asialoglycoprotein

receptor (ASGPR), an endocytic glycoprotein receptor, was also found in the group B genes.

Immune-related genes showed different responses to the pathogen (Table 3). The WSSV infection caused up-regulation of transcripts of MAC25 protein and heat shock protein70 at the late phase (72 hpi) whereas transcripts of the antimicrobial molecules, penaeidin and 11.5 kDa protein (crustin) were down-regulated after 24 hpi. Down-regulation of penaeidin and crustin was in accordance with those reported in the previous studies[25, 26, 27]. Injection of shrimp with *V. harveyei* as well as LPS caused a decrease in the gene expression of penaeidin and crustin. Such change in penaeidin expression has been shown to be due to the migration of penaeidin-producing haemoctes towards infected tissues and release of penaeidin, resulting in a marked decrease of penaeidin gene-expressing haemocytes in the circulating haemocyte population and an increase of penaeidin peptide concentrations in haemolymph[25]. Recently, Wang *et al.*[28] reported up-regulation of some immune genes as well as calcium-dependent genes in the cephalothorax of the chinese shrimp *Fenneropenaeus chinensis* infected with WSSV. These genes also include heat shock proteins similar to that found in our study and a calreticulin-like gene, a calcium-dependent gene that is a key regulator of calcineurin in the calcium-signaling pathway.

Other immune-related genes, prophenoloxidase activating factor (PPAF) and anti-lipopolysaccharide factor (ALF) did not respond to WSSV infection. The results correspond to Van de Braak *et al.*[29] which could not observed massive haemocytic aggregation and melanisation in WSSV-infected shrimp. Moreover, the inhibition of degranulation and the melanization suppression were found in WSSV-infected crayfish *Pacifastacus leniusculus* [30]. On the contrary, apoptosis might be implicated in shrimp death caused by WSSV [31]. Besides the changes in expression of these immune genes in shrimp haemocytes, several clones with unknown function were up-regulated throughout the pathogen infection (data not shown). These clones need to be further investigated for their roles and functions in the shrimp immune response.

The abundance of transcripts of calmodulin (CaM), ASGPR and tubulin increased about 20, 6 and 5 fold in response to WSSV infection (Table 3). CaM is a sensor protein of intracellular calcium fluxes that functions by binding to and regulated CaM binding proteins (CaMBPs) [32]. Identification of target proteins that bind CaM may help to elucidate signaling pathways that links CaM to the defense response in shrimp. Tubulin is the building block of microtubules, which are involved in many cellular processes such as cell division, cytoplasmic streaming, organelle positioning and signal transduction[33]. However, how tubulins are involved in the shrimp defense is not known. ASGPR is a calcium-dependent (C-type) animal lectin. It mediates endocytosis and degradation of serum proteins in mammalian hepatocytes[34] and is involved in the clearance of apoptotic bodies from the liver[35].

From the cDNA microarray results, the three up regulated genes in response to pathogen challenge, calmodulin (CaM), ASGPR and β-tubulin, were subjected to real-time PCR to accurately measure and verify their up regulated expression. Beside the elucidation of these up regulated genes in the viral challenged shrimp, the expression pattern of these 3 genes was also quantitatively determined in luminescence bacteria *V. harveyi* challenged shrimp. Results of the real-time PCR experiment are shown in Fig. 2. In WSSV-challenged shrimp, the CaM mRNA expression increased significantly after 24 hpi and the up-regulation was observed until 72 hpi (Fig. 2A). In the *V. harveyi*-challenged shrimp, the expression of the CaM gene significantly increased at 6 hpi, and rather highly expressed until 48 hpi. (Fig 2B). The tubulin

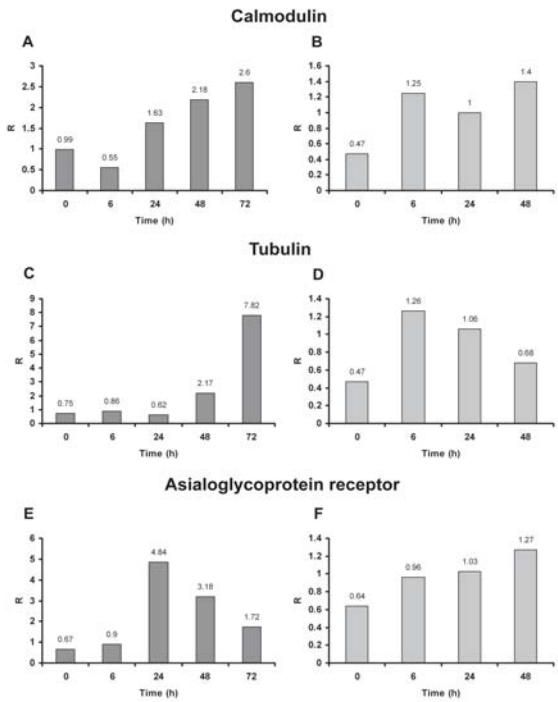

Fig 2. Relative expression patterns of calmodulin, tubulin and asialoglycoprotein receptor genes in haemocytes of *P. monodon* challenged with WSSV and *V. harveyi* using real-time PCR analysis. A: calmodulin (WSSV infection), B: calmodulin (*V. harveyi* infection), C: tubulin (WSSV infection), D: tubulin (*V. harveyi* infection), E: asialoglycoprotein receptor (WSSV infection), F: asialoglycoprotein receptor (*V. harveyi* infection). Shrimp haemocytes were collected at 0, 6, 24, 48 and 72 h post WSSV challenge and at 0, 6, 24 and 48 h post *V. harveyi* challenge.

mRNA expression did not increased in *P. monodon* haemocytes until 48 h after the WSSV injection and the highest expression was observed at 72 hpi (Fig. 2C). In the *V. harveyi* -challenged shrimp, the considerable up-regulation of the tubulin mRNA was observed at the early phase of bacterial injection (Fig. 2D). The ASGPR transcripts also showed significantly increased expression after viral and bacterial injection (Fig.2 E and F). The real-time PCR analysis showed that the genes encoding for CaM, tubulin and ASGPR were up-regulated in response to the pathogen challenge, in accordance with the results of the microarray analysis. Our results confirmed the involvement of CaM, ASGPR and tubulin in the shrimp response. It should be noted that the three genes showed similar expression pattern responding to viral and bacterial infections suggested that their responses may be involved the same regulatory pathway. Interestingly, each of these proteins is affected by the calcium ion[36,37]. The calcium ion is a fundamental intracellular messenger that is involved in many cellular pathways. Cell survival and apoptosis have been reported to be induced by cellular calcium ions[38]. However, additional studies are necessary to elucidate the mechanism responsible for their cellular pathways.

Several techniques have been used to identify differentially expressed genes including suppression-subtractive hybridization (SSH), differential display PCR (DD-PCR), serial analysis of gene expression (SAGE) and microarray analysis. Recently, we performed the DD-PCR to identify genes potentially responded to *V. harveyi* challenge in *P.monodon*. Several genes with known function were identified including those involve in immune function e.g. caspase3B, serpinB3, glucose transporter, lyzosyme, and anti-lipopolysaccharide factor[11]. Genes highly response to *V. harveyi* challenge found in the previous study were different from those responded to WSSV challenge reported in this study. This may indicate different immune responses of shrimp to different pathogens.

The DNA microarray approach provides an attractive solution for effectively identifying candidate genes involved in the pathogenesis of diseases. It provides high sensitivity for the detection of subtle differences that are much harder to detect with subtraction or other molecular methods. The technique could generate new hypotheses for mechanisms underlying the development of damage and might enhance our understanding in pathogenesis of diseases in the penaeid shrimp.

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