

Discovery of the Genes in Response to White Spot Syndrome Virus (WSSV) Infection in *Fenneropenaeus chinensis* Through cDNA Microarray

Bing Wang,^{1,2} Fuhua Li,¹ Bo Dong,¹ Xiaojun Zhang,¹ Chengsong Zhang,¹ Jianhai Xiang¹

¹Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Road, 266071 Qingdao, People's Republic of China

²Graduate School, Chinese Academy of Sciences, 100039 Beijing, People's Republic of China

Received: 20 September 2005 / Accepted: 9 February 2006 / Published online: 26 June 2006

Abstract

We used microarray technology to study differentially expressed genes in white spot syndrome virus (WSSV)-infected shrimp. A total of 3136 cDNA targets, including 1578 unique genes from a cephalothorax cDNA library and 1536 cDNA clones from reverse and forward suppression subtractive hybridization (SSH) libraries of *Fenneropenaeus chinensis*, plus 14 negative and 8 blank control clones, were spotted onto a 18 × 18 mm area of NH₂-modified glass slides. Gene expression patterns in the cephalothorax of shrimp at 6 h after WSSV injection and moribund shrimp naturally infected by WSSV were analyzed. A total of 105 elements on the arrays showed a similar regulation pattern in artificially infected shrimp and naturally infected moribund shrimp; parts of the results were confirmed by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The up-regulated expression of immune-related genes, including heat shock proteins (HSP70 and HSP90), trehalose-phosphate synthase (TPS), ubiquitin C, and so forth, were observed when shrimp were challenged with WSSV. Genes including myosin LC2, ATP synthase A chain, and arginine kinase were found to be down-regulated after WSSV infection. The expression of housekeeping genes such as actin, elongation factor, and tubulin is not stable, and so these genes are not suitable as internal standards for semiquantitative RT-PCR when shrimp are challenged by WSSV. As a substitute, we found that triosephosphate isomerase (TPI) was an ideal candidate of interstandards in this situation.

Keywords: Expressed sequence tags — *Fenneropenaeus chinensis* — microarray — subtractive sup-

pression hybridization library — white spot syndrome virus

Introduction

Chinese shrimp, *Fenneropenaeus chinensis*, is one of the most important mariculture species in China. Over the years, however, its culture industry has been seriously affected by white spot syndrome virus (WSSV) infection (Xiang, 2001). WSSV was first found in South Asia and then spread to America, Europe, and Australia (Krishna et al., 1997). The mortality rate of WSSV-infected shrimp was almost 100% in 3 to 10 days (van Hulst et al., 2001). Because of its rapid spread and high associated mortality rates, WSSV is an extremely virulent pathogen in shrimp culture. Although the entire genome sequencing of WSSV has been completed (van Hulst et al., 2001; Yang et al., 2001), and several WSSV structural protein genes have been identified via proteomic approaches (Huang et al., 2002; van Hulst et al., 2002). No effective cure for this disease has yet been found.

Shrimp have no acquired adaptive immune system; their defense is believed to depend entirely on an innate, nonadaptive mechanism to resist invasion by pathogens (Gross et al., 2001) and inoculation against viruses has no effect. Understanding the interaction between host and pathogen will be helpful in controlling infectious diseases in shrimp. Although an increasing number of immune function related genes of shrimp have been reported, including antilipopolysaccharide factor (Liu et al., 2005), penaeidin-like antimicrobial peptide (Chiou et al., 2005), and kazal-type serine proteinase inhibitor (Jarasrassamee et al., 2005), the genes involved in the interaction between WSSV and shrimp still

Correspondence to: Jianhai Xiang; E-mail: jhxiang@ms.qdio.ac.cn

remain unclear. Knowledge about shrimp functional genomics would enable detailed investigation of the shrimp genes that are involved in the pathogenesis of WSSV infection.

Obtaining expressed sequence tags (ESTs) by partial sequencing of cDNA libraries is an effective means of discovering new genes in organisms for which genomic data are unavailable (Adams et al., 1991). To gain more information on the genomics of *Fenneropenaeus chinensis*, more than 10,000 ESTs have been generated and analyzed from a cephalothorax cDNA library. A total of 3120 unique genes, including 1399 contigs and 1721 singletons, were generated in our laboratory (Xiang et al., 2002), and many immune-related factors were discovered by annotation of ESTs (Shen et al., 2004). Subtractive suppression hybridization (SSH) technology combines normalization and subtraction in a single procedure based primarily on suppression polymerase chain reaction (PCR) (Diatchenko et al., 1996) and allows the isolation of differentially expressed cDNAs with scarcely genomic sequences (Munir et al., 2004). Forward and reverse SSH libraries were previously constructed from cephalothorax of shrimp 6 h after WSSV injection in our laboratory to facilitate research on differentially expressed genes between WSSV-infected and healthy shrimp.

Many genes in shrimp may be involved in the interaction between host and virus when the shrimp is infected by virus. Studies of the changes in gene expression profile can help us to identify the genes that play an important role in the shrimp antiviral system. The microarray technique has proved to be a powerful tool to investigate the expression of thousands of genes in a single hybridization (Schena et al., 1995). The cDNA microarray was used to study differentially expressed genes in both tissues and cell culture systems (Tsoi et al., 2003); SSH coupled with microarray has been successfully used together for studying gene expression profiles in various systems (Yang et al., 1999). Moreover, microarray technology was also successfully used on the marine species *Oncorhynchus keta* (Moriya et al., 2004), *Fundulus heteroclitus* (Oleksiak et al., 2001), *Karenia brevis* (Lidie et al., 2005), and *Salmo salar* (Tsoi et al., 2003), among others. Dhar et al. (2003) constructed a microarray of 100 elements including 84 different cDNA fragments of hepatopancreas from *Penaeus stylirostris* and found some differentially expressed genes involved in WSSV infection through microarray hybridization.

Using a batch of EST sequences and SSH libraries of WSSV-infected shrimp owned by us, we constructed a cDNA microarray including 3136 spots to investigate the differential expression of

genes in *Fenneropenaeus chinensis*. Because most of the important organs of shrimp, including hepatopancreas, heart, gills, stomach, and lymphoid organ, are located in the cephalothorax, cephalothorax was chosen as the target section of the body in this research.

Materials and Methods

Preparation of the Microarray. A low-density microarray of 3136 elements was constructed. The genes spotted onto the microarray included 1578 unique genes from the cephalothorax cDNA library, 1536 cDNA clones from reverse and forward SSH libraries, and 14 negative (Rice U2 RNA) and 8 blank (spotting solution) controls. Information about this array, including the accession number of ESTs, has been submitted to the NCBI GEO (accession number is GPL930). The clones of the unique genes from ESTs were selected based on bioinformatic analysis of the cephalothorax ESTs (Xiang et al., 2002). The longest ESTs of these unique genes were chosen. Forward and reverse SSH libraries were previously constructed from cephalothorax of the shrimp 6 h after WSSV injection via the PCR-Select™ cDNA Subtraction Kit (Clontech). Tissues from shrimp at 6 h post-injection of WSSV were selected to construct the SSH libraries because the immune-related genes showed a great immune response to stimulant approximately 6 h after the challenge (Liu et al., 2005). The clones from SSH libraries were selected randomly, and the cDNA fragments for these clones were amplified by PCR.

To obtain qualified cDNA fragments to construct the microarray, the PCR products were purified and quantitated by gel electrophoresis. The qualified products were then dissolved in 3× saline sodium citrate (SSC) solution and spotted onto a 18 × 18 mm area of NH₂-modified glass slides (Biostar) according to Schena et al. (1995). After the spotting, the slides were hydrated for 2 h and then dried at room temperature for 30 min, UV cross-linked (65 mJ/cm), and treated with 0.2% sodium dodecyl sulfate (SDS) and 0.2% NaBH₄ for 10 min each. The slides were then dried and stored for use. Construction of the microarray was performed at Biostar Gene Chip, Shanghai, China, via Cartesian 7500 Spotting Robotics (Cartesian).

Source of Samples for Microarray Hybridization. Two groups of samples were set for hybridization, each containing experimental shrimp and control. In group I, the experimental shrimp were injected with tissue homogenate isolated from WSSV-

infected shrimp, while the control shrimp were injected with tissue homogenate isolated from WSSV-free shrimp. To prepare the tissue homogenate for challenge, 10-g tissues of WSSV-infected or -free shrimp were homogenized separately in 10 ml phosphate-buffered saline (PBS)-His (Huang et al., 1999) on ice. The homogenized tissue was centrifuged at 3800 g at 4°C for 15 min. The supernatant was transferred to a fresh tube, sucrose was added to 30% (wt/wt), and then the mixture was centrifuged at 38,000 g at 4°C for 60 min. The pellets were resuspended in 10 ml of PBS-His buffer for injection. Each shrimp was injected with 3 µl of the above described tissue homogenate. After challenge, WSSV-specific PCR reaction was used to check the infection result. Both experimental and control shrimp were sacrificed at 6 h post-injection of WSSV. In group II, moribund shrimp that were naturally infected by WSSV in a culture pond were chosen as the experimental shrimp while WSSV-free wild shrimp from the Yellow Sea was taken as the control. Cephalothorax of three individuals from experimental or control shrimp of each group were placed in liquid nitrogen and triturated in a motor homogenizer. About 500 mg of triturated tissues were removed for RNA extraction. The integrity and purity of total RNA extracted from the tissues were assessed by gel electrophoresis.

Microarray Analysis. Fluorescence-labeled cDNA probe molecules were generated during first-strand cDNA synthesis using total RNA extracted from experimental and control shrimp in both groups. The probes from the control tissues were labeled with Cy3-dCTP while those from the experimental ones were labeled with Cy5-dCTP. The fluorescent cDNA probes were purified using the Qiagen PCR Purification Kit following the manufacturer's protocol. Hybridization experiments were performed following the procedure specified on the microarray hybridization kit (Biostar), and the hybridization experiment was repeated once for each group. After hybridization, the fluorescence intensities of the Cy5 and Cy3 signals of each target element were measured by scanning the slides on a

ScanArray 4000 scanner (General Scanning). The acquired images were analyzed via the software ImaGene 3.0, and the signal intensity of each element was normalized with a total intensity coefficient.

Semiquantitative RT-PCR. To confirm the microarray hybridization results, the expression of three genes including TPI (triosephosphate isomerase), HSP70, and TPS (trehalose-phosphate synthase) was detected through semiquantitative RT-PCR during WSSV challenge. The samples used for semiquantitative RT-PCR came from a batch used in an artificial challenge experiment and were different from the samples used in microarray hybridization. The preparation of tissue homogenate for injection followed the procedure described in the preceding text, and the injection dose was also 3 µl per shrimp while the source of shrimp was different. Hepatopancreas and lymphoid organs of nine individuals were dissected from shrimp challenged 6 h post-injection of WSSV and from moribund shrimp after challenge for RT-PCR detection. At the same time, shrimp tissues were removed from the control group. Hepatopancreas or lymphoid organs from three individuals were pooled and three pools for experimental or control shrimp were used for RNA extraction with Trizol reagent (Invitrogen) following the manufacturer's protocol. The cDNA was synthesized in a 25-µl reaction volume containing 2 µg of DNase I-treated total RNA, 1× Moloney Murine Leukemia Virus (MMLV) buffer, 0.5 mM dNTP, 0.4 mM oligo-dT, 20 U of RNase inhibitor (Promega), and 200 U of MMLV reverse transcriptase (Promega). The cDNA was then diluted to 1:5, and 1 µl of the dilution was used for each RT-PCR reaction. The amplifications were performed in a 25-µl reaction volume containing 1× PCR buffer, 1.75 mM MgCl₂, 0.2 mM dNTP, 0.4 mM of forward and reverse primers, and 1 µl of 1:5 diluted cDNA. The primers used for RT-PCR assay are listed in Table 1. The thermal profile for RT-PCR was 95°C for 5 min followed by 24 cycles of 95°C for 40 s, 60°C for 1 min, 72°C for 1 min, and finally 72°C for 10 min. The products (3 µl/lane) were detected on 1.0% agarose.

Table 1. Primers Used in Semiquantitative RT-PCR

<i>Gene name</i>	<i>Primer sequence</i>	<i>T_m (°C)</i>	<i>Size of PCR products</i>
Triosephosphate isomerase (TPI)	5'-GGTGGAAACTGGAAGATGAACG 5'-ACAAAGTCTGGCTTGAGTGATG	60°C	696 bp
Trehalose-phosphate synthase (TPS)	5'-GTCCAGCATCGGAAACAACCTC 5'-GACGCACGGTCAATACAACAG	60°C	387 bp
Heat shock protein 70 (HSP70)	5'-TCAAAATCGTCTCACTCCT 5'-TTTACCCAACCTCTCACACAG	50	532 bp

Table 2. Differentially Expressed Genes from ESTs with the Same Regulation Tendency in Two Groups of Hybridization

GeneID	Definition	C _γ 5/C _γ 3 ratio	
		Group I (6-h)	Group II (moribund)
<i>Cell growth and maintenance</i>			
C1285	G2/mitotic-specific cyclin B	14.4295	4.7415
C989	G2/mitotic-specific cyclin B	7.1645	2.2105
C1166	Tubulin β1 chain	6.5935	2.703
C624	α-Aspartyl dipeptidase	2.8735	4.5335
<i>Histogenesis and organogenesis</i>			
C1231	Myosin light chain 2	0.1855	0.2285
<i>Metabolism and homeostasis</i>			
C982	Protein transport protein sec61 γ subunit	4.432	2.8575
392	MNN4	0.4855	0.325
C930	ATP synthase A chain	0.378	0.346
<i>Known or potential immune function</i>			
C1298	Heat shock 70-kDa protein cognate 3 precursor	25.354	4.1335
C760	Trehalose-phosphate synthase	15.613	2.389
C1327	Calreticulin precursor (CRP55)	15.0295	5.627
C1168	Heat shock protein HSP 90α	8.382	4.0025
314	Thioredoxin	6.029	9.83
C1290	Protein disulfide isomerase	4.07	5.3105
<i>RNA replication, repair, translation, and transcription that includes rRNA</i>			
575	Splicing factor, arginine /serine-rich 4	5.7185	64.38
C653	Probable eukaryotic translation initiation factor	5.4655	115.1125
C993	Cyclic-AMP-dependent transcription factor	5.0735	7.6395
C1253	<i>Rattus norvegicus</i> ubiquitin C [Ubc]	2.847	2.9525
442	Small nuclear ribonucleoprotein polypeptide G	2.7695	5.8785
<i>Putative receptor, signal transduction</i>			
C1280	Mucin 2 precursor	5.008	103.21
<i>Unknown</i>			
C545	CG6084 gene product	5.7375	2.313
C877	CG7415 [<i>Drosophila melanogaster</i>] (AY051625)	4.6405	2.3275
C1044	Lark (U59476) Lark RNA	3.7345	9.2945
C714	Developmental embryonic B	3.067	2.698
C1076	Early E3B 14.5-kDa protein precursor	2.46	4.001
<i>Group II (moribund)</i>			
Mitosis			
Mitosis			
Microtubule-based process			
mitosis			
Calcium binding			
Protein targeting			
Carbohydrate metabolism			
ATP synthesis, proton transport			
Heat shock response			
Trehalose biosynthesis			
Calcium binding			
Heat shock response			
Electron transport			
Electron transport			
mRNA processing			
Translational regulation			
Transcription regulation			
Gene silencing			
mRNA processing			
Cell-matrix adhesion			
Oxidoreductase activity			
Dipeptidyl-peptidase III activity			
Negative regulation of eclosion			
mRNA processing			
Unknown			

Results

The hybridization showed high specificity for shrimp cDNA because negative (Rice U2 RNA) and blank (spotting solution) targets lack a hybridization signal. Average signal intensities of hybridization were three times above background, and the intensity coefficient of the microarray hybridization was between 0.4 and 2.5, indicating that the hybridization results are reliable. The standards for differentially expressed elements are: intensity value of Cy3 and Cy5 being larger than 200 or one of them being above 800, and the ratio of Cy5/Cy3 being above 2 or under 0.5.

Among 3114 elements analyzed, the differentially expressed elements in the shrimp tissues at 6 h post-WSSV injection (group I) were 1019; those in tissues of moribund shrimp spontaneously infected with WSSV were 305. Among the above elements that showed differential expression, 105 showed a similar expression profile in both artificially challenged (group I) and spontaneously infected shrimp (group II). Among the 105 elements, 51 were from the unique genes of ESTs while the remaining 54 were from the clones of SSH libraries.

Differentially Expressed Unique Known Genes from the EST Database. Of the 51 unique genes of EST, 25 were known genes and the remaining 26 were unknown ones. The 25 known genes that

showed similar expression patterns in the two groups of WSSV-infected shrimps are presented in Table 2; 22 of these were up-regulated and 3 were down-regulated. The up-regulated genes included HSP70, HSP90, trehalose-phosphate synthase, and ubiquitin C. The gene up-regulated ratios were different from each other between these two groups. Trehalose-phosphate synthase is one of the strongest up-regulated genes among the immune-related genes. The down-regulated known genes were myosin light chain (MLC), ATP synthase, and MNN4. The expression of housekeeping genes such as β -actin, elongation factor, and tubulin was unstable during WSSV infection.

Differentially Expressed Clones of SSH Libraries. The 52 differentially expressed clones from SSH libraries were screened and sequenced. The sequences were blasted against the NCBI database. The total number of matched differentially expressed clones was 43 and that of unmatched ones was 9. However, 26 sequences matched with WSSV sequences and so they were deleted from the record. Among other matched clones, only 1 clone was up-regulated and 16 were down-regulated (Table 3). The β 1-tubulin was the only up-regulated shrimp gene acquired from SSH libraries while the arginine kinase, actin, phosphopyruvate hydratase, ATP synthase β -subunit, nucleoside diphosphate

Table 3. Differentially Expressed Genes from SSH Libraries with the Same Regulation Tendency in Two Groups of Hybridization

Gene ID	Definition	Cy5/Cy3 ratio		Molecular function
		Group I (6-h)	Group II (moribund)	
<i>Cell growth and maintenance</i>				
R3(G1)	α 1-tubulin	9.5555	3.8365	Microtubule-based process
<i>Histogenesis and organogenesis</i>				
F2(H12)	Actin	0.3845	0.3705	Cytoskeleton organization and biogenesis
F3(F12)	Actin	0.213	0.3655	Cytoskeleton organization and biogenesis
<i>Metabolism and homeostasis</i>				
R1(F10)	Arginine kinase (AK)	0.406	0.1315	Energy pathways
R3(E9)	Arginine kinase (AK)	0.3945	0.26	Energy pathways
F1(F9)	Nucleoside diphosphate kinase	0.3655	0.096	Protein biosynthesis
R3(E2)	Arginine kinase (AK)	0.3225	0.175	Energy pathways
Fs3(B8)	Phosphopyruvate hydratase	0.3205	0.1415	Glycolysis
F4(H10)	Phosphopyruvate hydratase	0.3005	0.215	Glycolysis
F5(D6)	Phosphopyruvate hydratase	0.2985	0.0805	Glycolysis
R1(E1)	Hemocyanin	0.2565	0.176	Metabolism
R5(C8)	EF2Mc2 elongation factor 2 (Ef-2)	0.254	0.2405	Protein biosynthesis
R4(B1)	EF2Mc2 elongation factor 2 (Ef-2)	0.2405	0.363	Protein biosynthesis
R4(H6)	Arginine kinase (AK)	0.1795	0.286	Energy pathways
R1(C1)	Arginine kinase (AK)	0.086	0.236	Energy pathways
R1(F1)	Arginine kinase (AK)	0.0845	0.1775	Energy pathways
F3(C5)	Phosphopyruvate hydratase	0.0805	0.127	Glycolysis

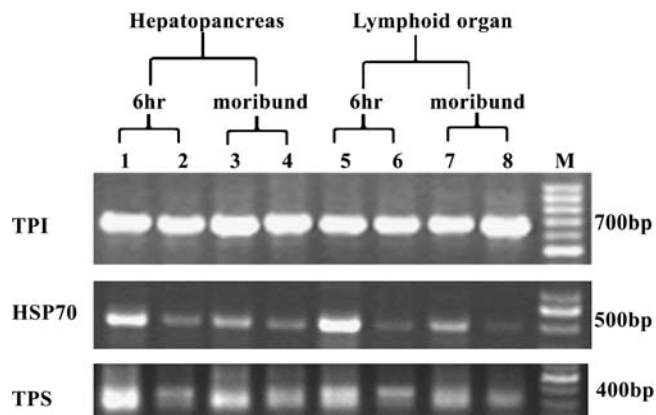


Fig. 1. Expression profiles of HSP70 and TPS genes in hepatopancreas and lymphoid organ during WSSV infection. *Lane 1*, hepatopancreas of shrimp at 6 h post-injection of WSSV; *lane 2*, hepatopancreas of control shrimp at the same time; *lane 3*, lymphoid organ of shrimp at 6 h post-injection of WSSV; *lane 4*, hepatopancreas of control shrimp at 6 h post-injection; *lane 5*, hepatopancreas of moribund shrimp after WSSV infection; *lane 6*, hepatopancreas of shrimp as a control of moribund shrimp; *lane 7*, lymphoid organ of moribund shrimp after WSSV infection; *lane 8*, lymphoid organ of shrimp as a control of moribund shrimp.

kinase, elongation factor, and hemocyanin were down-regulated.

With microarray hybridization, we have proved that the expressions of chaperones, calcium-dependent genes, cell structure genes, energy metabolism genes, and some housekeeping genes were altered as a result of WSSV infection.

Semiquantitative RT-PCR Detection. RT-PCR detection showed that the shrimp TPI gene was expressed stably in hepatopancreas, lymphoid organ, blood, muscle, gills, and gut during WSSV infection, while the HSP70 and TPS genes were up-regulated in hepatopancreas and lymphoid organ of both 6-h post-injection shrimp and moribund shrimp, which is consistent with our microarray hybridization results. The expression of HSP70 and TPS genes in the tissues of the 6-h post-injection shrimp was higher than that in the moribund shrimps (Figure 1), which greatly supports the microarray hybridization results.

Discussion

Although the infection methods in the two groups were different—one was artificially challenged shrimp and the other shrimp spontaneously infected with WSSV in a farm tank—the common characteristic for these two groups is WSSV infection. We paid special attention to the genes that showed the

same expression patterns between the two groups, and the accuracy of the results should be greatly increased. Two of the most significantly up-regulated genes and one stably expressed gene found through microarray hybridization were further confirmed in another WSSV challenge experiment via semiquantitative RT-PCR.

The innate immune system is the first line of defense to protect the host in the first hours to days of infection (Lee and Soderhall, 2002). The first distinct phase of the immune response in shrimp is approximately in the first 12 h after challenge (Bachere et al., 2004). The mRNA level of an immune function related gene, C-type lectin-1, starts to show differential expression 2 h post-WSSV-injection (Dhar et al., 2003). Liu et al. (2005) found that the expression of ALF increased strikingly in the first 6 h after the pathogen challenge. Based on the consideration that 6 h post-challenge might be an important stage when expression of shrimp immune genes varies greatly, we chose shrimp tissues at 6 h post-injection for microarray hybridization.

There are two categories of genes to which we should pay more attention. The first are genes that perform cell growth and immune functions. Their expressions were up-regulated in both tissues of 6-h post-infected shrimp and moribund shrimp, but their regulation ratios were not the same between the two experimental groups. In the tissues of moribund shrimp, the up-regulation ratios of most of those genes, such as tubulin, cyclin B, trehalose-phosphate synthase, HSP70, HSP90, and calreticulin, were lower than those of 6-h post-injection of WSSV (Tables 2 and 3). Another category comprises the genes that are involved in metabolism and homeostasis. Most of those genes, such as AK, ATP synthase A chain, MNN4, phosphopyruvate hydratase, hemocyanin, and elongation factor, were down-regulated significantly. The expression of these genes may indicate that the infection with WSSV could cause a malfunction of metabolism and the immune system in shrimp.

Trehalose-Phosphate Synthase. Interestingly, the expression intensity of TPS in shrimp tissues 6 h post-injection of WSSV increased about 15 times over that of the control. TPS is a key enzyme of trehalose biosynthesis. Trehalose is a disaccharide formed by a 1,1-linkage of two D-glucose molecules (Birch, 1963). Reportedly, trehalose can protect the integrity of cells against environmental stresses such as desiccation, dehydration, heat, cold, and oxidation (Strom and Kaasen, 1993; Block, 2003; Chen and Haddad, 2004). High-level expression of

the TPS gene in shrimp tissues 6 h post-injection of WSSV implies that TPS plays an important role in the immune system of shrimp against viral invasion. The present results form the first report suggesting the involvement of this gene in WSSV infection.

Chaperones. HSP70 and HSP90 function as chaperones, correcting folding of proteins, repairing DNA, and forming multiprotein assemblies (Bukau and Horwich, 1998; Kumar et al., 2003). In this microarray experiment, not only HSP70 and HSP90, but also the chaperones protein disulfide isomerase and calreticulin precursor were up-regulated in WSSV-infected tissues. The results showed that the chaperone proteins are active in defending against WSSV infection.

Calcium-Dependent Genes. In this microarray hybridization, we found several calcium-related factors that are differentially expressed in WSSV-infected shrimp. Obviously WSSV can affect calcium cycling and damage the cytoskeleton and the immune system of the host. Major factors involved were calreticulin, myosin light chain, and DD9A. Among them, the up-regulation of the calreticulin-like gene was more noticeable. Calreticulin is one of the chaperones in endoplasmic reticulum (ER), a component of the ER quality control system (Leach and Williams, 2004; Molinari et al., 2004) and also a key upstream regulator of calcineurin in the calcium-signaling pathway (Lynch and Michalak, 2003; Groenendyk et al., 2004). The up-regulation of calreticulin may also be a compensating reaction to the calcium cycling in WSSV infection.

It is very interesting that the expression of the cuticle protein, DD9A, was down-regulated in shrimp tissue 6 h post-injection of WSSV and terminated in the moribund shrimp. DD9A was reported to participate in calcification of the shrimp exoskeleton (Watanabe et al., 2000). The down-regulation of DD9A may explain abnormal deposits of calcium salts, which were usually called white spots on the shells of WSSV-infected shrimp. We deduced that WSSV could alter the calcium cycling of the host. Calcium is a universal messenger of intracellular signaling for a wide variety of cell processes (Harnett and Biancani, 2003), and several immune related factors are calcium-dependent such as transglutaminases (Lorand and Conrad, 1984), calcium-dependent lectin (Kimura et al., 1995), collectin (Holmskov, 2000), calcineurin (Rao, 1994), and the calcium-independent class of protein kinase C (Nishizuka, 1992). In a study on lymphoid cells of advanced animals, proliferation of the lymphoid cell induced

by cross-linked anti-CD3 mAb or by Con A was markedly depressed, and this suppression was associated with a reduction in the influx of calcium (Haque et al., 1998). Hence we inferred that this kind of regulation is fatal to the shrimp, and the potential roles of the DD9A protein and calcium in WSSV infection should be studied in detail.

MLC and MNN4 were found down-regulated in this microarray experiment. MLC is one of the components of myosin and has the property of calcium binding. The phosphorylation and dephosphorylation of MLC is calcium-dependent (Somlyo et al., 1999). Generally, myosin act as molecular motors and interaction partners of actin filaments. Because myosin is involved in the reaction of antigen presentation (Wulfing and Davis, 1998), down-regulation of myosin may weaken the immune system of shrimp. We also found that the expression of some genes encoding antimicrobial peptide, peritrophin-like protein, and cuticle proteins were terminated in the moribund tissues (data not shown). Down-regulation of these genes may destroy the immune reaction of the host because they participate in the defense function directly or indirectly. MNN4, which is likely to function as a positive regulator of mannosylphosphate transferase (Odani et al., 1996), was down-regulated during WSSV infection. MNN4 might be involved in the cellular response to a variety of stresses (Odani et al., 1997), and hence its down-regulation might cause a negative effect on the shrimp defense system.

Cell Structure and Energy Metabolism Factors. The down-regulated genes include genes involved in cell structure and energy metabolism such as arginine kinase (AK), actin, phosphopyruvate hydratase, ATP synthase β -subunit, nucleoside diphosphate kinase, elongation factor, and hemocyanin (Table 3). These data indicated that the WSSV has restrained the expression of host genes, which must have impaired the immune system of the shrimp.

It is interesting to note that the clones similar to arginine kinase (AK) were down-regulated significantly. AK is one of the shrimp allergens homologous to arginine kinase in all crustaceans (Bernstein et al., 1982; Yu et al., 2003), and was reported as a phosphagen-ATP phosphotransferase (Hird, 1986). This result is different from the report of Astrofsky et al. (2002), who found that AK was up-regulated after 30 h of WSSV injection. Astrofsky et al. (2002) took the hepatopancreas tissue 30 to 40 h post-injection of WSSV for the experiment and used the EF- α as an interstandard, so that the differential infection phase and interstandard might be the

main reason for this difference. We have deduced that the expression pattern of AK might fluctuate at different stages of WSSV infection. Because AK plays an important role in energy metabolism (Dumas and Camonis, 1993), it is thought that the down-regulation might reflect damage of energy metabolism. Another energy metabolism enzyme, ATP synthase, was also found down-regulated. The down-regulation of cell structure proteins and energy metabolism factors certainly affected the response ability, such as the immune reaction, of the host shown in our study.

The clone similar to β 1-tubulin was a sole up-regulated gene acquired from SSH library. β 1-tubulin is a major cytoskeleton component that participates in the formation of microtubule filament (Burns, 1991; Luduena, 1998). Research on viruses of insects indicated that the microtubule filament could take part in the invasion and transportation of viruses (Talhouk and Volkman, 1991), but the function of β 1-tubulin in WSSV pathogenesis is unknown.

Viruses have proved to have the ability to regulate host cellular protein synthesis to their own advantage (Schneider and Mohr, 2003). Although it is certain that WSSV infection causes tissue damage, it is not clear why this infection affects the energy metabolism of the host. Some host genes, including splicing factor, mucin, and eukaryotic translation initiation factor, were utilized by WSSV. We noted that the expression level of these genes in tissues of moribund shrimp was significantly higher than that in tissues of shrimp at 6 h post-injection of WSSV. The phenomenon was probably caused by the action of WSSV, because the viruses were active for replication. Thus these genes were probably involved in the course of WSSV infection as cooperating factors. A high expression level of splicing factor and translation initiation factor indicated that RNA export was active (Jefferson and Kimball, 2003). As for mucin, most research in higher animals reported that the quality and quantity of mucin expression changed under pathological conditions (Ringel and Lohr, 2003). High expression levels of mucin in the moribund tissues suggested that this protein could play a cooperating role in the late phase of WSSV infection. Further investigation is needed on the function and characteristics of cell structure and energy metabolism factors during WSSV infection.

Housekeeping Genes. β -actin and elongation factor are the most frequently used internal standard in RT-PCR. But in our microarray results, the expression of housekeeping genes such as β -actin and elongation factor was unstable during

WSSV infection. Variation of expression of these housekeeping genes may affect the reliability of RT-PCR results when they were used as internal standards. Therefore these housekeeping genes may not be suitable for this standard in experiments on WSSV infection, and we need a stably expressed gene as internal standard for semiquantitative RT-PCR. By using semiquantitative RT-PCR, we found that TPI was stably expressed not only in cephalothorax but also in hepatopancreas, lymphoid organ, blood, muscle, gills and gut, implying that this gene can be an ideal candidate internal standard for gene quantitative analysis during WSSV invasion.

Conclusion

Our study indicated that WSSV infection could alter the expression of host genes in a wide range, especially in the early stage of infection. The differential expressions of calcium binding proteins indicate the possibility that the infection is directed toward host calcium cycling, which might then affect the calcium-mediated signal pathway in shrimp. We also found that the WSSV could destroy the host cytoskeleton and shut down the genes involved in host energy metabolism. These findings show that WSSV is able to switch off or limit the expression of the host's immune-related genes to facilitate its own replication in the host.

Acknowledgments

We thank Professor P. Natarajar, TWAS-UNESCO, and Dr. Ka Hou Chu, Chinese University of Hong Kong for their constructive comments on this manuscript. This work was supported by the Key Program of National Natural Science Foundation of China (30230280) and the National High Technology Research and Development Program (2005AA626014).

References

- Adams MD, Kelley JM, Gocayne JD, Dubnick M, Polymeropoulos MH, Xiao H, Merrill CR, Wu A, Olde B, Moreno RF (1991) Complementary DNA sequencing: expressed sequence tags and human genome project. *Science* 252, 1651–1656
- Astrofsky KM, Roux MM, Klimpel KR, Fox JG, Dhar AK (2002) Isolation of differentially expressed genes from white spot virus (WSV) infected Pacific blue shrimp (*Penaeus stylirostris*). *Arch Virol* 147, 1799–1812

- Bachere E, Gueguen Y, Gonzalez M, de Lorgeril J, Garnier J, Romestand B (2004) Insights into the anti-microbial defense of marine invertebrates: the penaeid shrimps and the oyster *Crassostrea gigas*. *Immunol Rev* 198, 149–168
- Bernstein M, Day JH, Welsh A (1982) Double-blind food challenge in the diagnosis of food sensitivity in the adult. *J Allergy Clin Immunol* 70, 205–210
- Birch GG (1963) "Trehaloses." In: *Advances in Carbohydrate Chemistry*, Vol. 18, Wolfson ML, Tipson IS, eds. (New York: Academic Press), pp 201–225
- Block W (2003) Water or ice?—the challenge for invertebrate cold survival. *Sci Prog* 86, 77–101
- Bukau B, Horwich AL (1998) The Hsp70 and Hsp60 chaperone machines. *Cell* 92, 351–366
- Burns RG (1991) Alpha- beta- and gamma-tubulins: sequence comparisons and structural constraints. *Cell Motil Cytoskeleton* 20, 181–189
- Chen Q, Haddad GG (2004) Role of trehalose phosphate synthase and trehalose during hypoxia: from flies to mammals. *J Exp Biol* 207, 3125–3129
- Chiou TT, Wu JL, Chen TT, Lu JK (2005) Molecular cloning and characterization of cDNA of penaeidin-like antimicrobial peptide from tiger shrimp (*Penaeus monodon*). *Mar Biotechnol* 7, 119–127
- Dhar AK, Dettori A, Roux MM, Klimpel KR, Read B (2003) Identification of differentially expressed genes in shrimp (*Penaeus stylirostris*) infected with White spot syndrome virus by cDNA microarrays. *Arch Virol* 148, 2381–2396
- Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc Natl Acad Sci USA* 93, 6025–6030
- Dumas C, Camonis J (1993) Cloning and sequence analysis of the cDNA for arginine kinase of lobster muscle. *J Biol Chem* 268, 21599–21605
- Groenendyk J, Lynch J, Michalak M (2004) Calreticulin, Ca²⁺, and calcineurin - signaling from the endoplasmic reticulum. *Mol Cells* 17, 383–389
- Gross PS, Bartlett TC, Browdy CL, Chapman RW, Warr GW (2001) Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific white shrimp, *Litopenaeus vannamei*, and the Atlantic white shrimp, *L. setiferus*. *Dev Comp Immunol* 25, 565–577
- Haque S, Dumon H, Haque A, Kasper LH (1998) Alteration of intracellular calcium flux and impairment of nuclear factor-AT translocation in T cells during acute *Toxoplasma gondii* infection in mice. *J Immunol* 161, 6812–6818
- Harnett KM, Biancani P (2003) Calcium-dependent and calcium-independent contractions in smooth muscles. *Am J Med (Suppl)* 115, 24–30
- Hird FJ (1986) The importance of arginine in evolution. *Comp Biochem Physiol B* 85, 285–288
- Holmskov UL (2000) Collectins and collectin receptors in innate immunity. *APMIS (Suppl)* 100, 1–59
- Huang C, Zhang X, Lin Q, Xu X, Hew CL (2002) Characterization of a novel envelope protein (VP281) of shrimp white spot syndrome virus by mass spectrometry. *J Gen Virol* 83, 2385–2392
- Huang J, Song XL, Yu J, Zhang LK (1999) The components of an inorganic physiological buffer for *Penaeus chinensis*. *Methods Cell Sci* 21, 225–230
- Jarasrassamee B, Supungul P, Panyim S, Klinbunga S, Rimphanichayakit V, Tassanakajon A (2005) Recombinant expression and characterization of five-domain Kazal-type serine proteinase inhibitor of black tiger shrimp (*Penaeus monodon*). *Mar Biotechnol* 7, 46–52
- Jefferson LS, Kimball SR (2003) Amino acids as regulators of gene expression at the level of mRNA translation. *J Nutr* 133, 2046–2051
- Kimura T, Imai Y, Irimura T (1995) Calcium-dependent conformation of a mouse macrophage calcium-type lectin. Carbohydrate binding activity is stabilized by an antibody specific for a calcium-dependent epitope. *J Biol Chem* 270, 16056–16062
- Krishna RR, Rao KG, Rao P, Babu PH (1997) White spot disease. *World Aquacult* 12, 14–19
- Kumar Y, Chawla A, Tatu U (2003) Heat shock protein 70 as a biomarker of heat stress in a simulated hot cockpit. *Aviat Space Environ Med* 74, 711–716
- Leach MR, Williams DB (2004) Lectin-deficient calnexin is capable of binding class I histocompatibility molecules *in vivo* and preventing their degradation. *J Biol Chem* 279, 9072–9079
- Lee SY, Soderhall K (2002) Early events in crustacean innate immunity. *Fish Shellfish Immunol* 12, 421–437
- Lidie KB, Ryan JC, Barbier M, Van Dolah FM (2005) Gene expression in Florida Red tide dinoflagellate *Karenia brevis*: analysis of an expressed sequence tag library and development of DNA microarray. *Mar Biotechnol* 7, 481–493
- Liu F, Liu Y, Li F, Dong B, Xiang J (2005) Molecular cloning and expression profile of putative antilipopolysaccharide factor in Chinese shrimp (*Fenneropenaeus chinensis*). *Mar Biotechnol* 7, 600–608
- Lorand L, Conrad SM (1984) Transglutaminases. *Mol Cell Biochem* 58, 9–35
- Luduena RF (1998) Multiple forms of tubulin: different gene products and covalent modifications. *Int Rev Cytol* 178, 207–275
- Lynch J, Michalak M (2003) Calreticulin is an upstream regulator of calcineurin. *Biochem Biophys Res Commun* 311, 1173–1179
- Molinari M, Eriksson KK, Calanca V, Galli C, Cresswell P, Michalak M, Helenius A (2004) Contrasting functions of calreticulin and calnexin in glycoprotein folding and ER quality control. *Mol Cell* 13, 125–135
- Moriya S, Urawa S, Suzuki O, Urano A, Abe S (2004) DNA microarray for rapid detection of mitochondrial DNA haplotypes of chum salmon. *Mar Biotechnol* 6, 430–434
- Munir S, Singh S, Kaur K, Kapur V (2004) Suppression subtractive hybridization coupled with microarray analysis to examine differential expression of genes in virus infected cells. *Biol Proc Online* 6, 94–104
- Nishizuka Y (1992) Intracellular signaling by hydrolysis of

- phospholipids and activation of protein kinase C. *Science* 258, 607–614
- Odani T, Shimma Y, Tanaka A, Jigami Y (1996) Cloning and analysis of the MNN4 gene required for phosphorylation of N-linked oligosaccharides in *Saccharomyces cerevisiae*. *Glycobiology* 6, 805–810
- Odani T, Shimma Y, Wang XH, Jigami Y (1997) Mannosylphosphate transfer to cell wall mannan is regulated by the transcriptional level of the MNN4 gene in *Saccharomyces cerevisiae*. *FEBS Lett* 420, 186–190
- Oleksiak MF, Kolell KJ, Crawford DL (2001) Utility of natural populations for microarray analyses: isolation of genes necessary for functional genomic studies. *Mar Biotechnol Suppl*, S203–211
- Rao A (1994) NF-ATp: a transcription factor required for the co-ordinate induction of several cytokine genes. *Immunol Today* 15, 274–281
- Ringel J, Lohr M (2003) The MUC gene family: their role in diagnosis and early detection of pancreatic cancer. *Mol Cancer* 2, 9
- Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270, 467–470
- Schneider RJ, Mohr I (2003) Translation initiation and viral tricks. *Trends Biochem Sci* 28, 130–136
- Shen YQ, Xiang JH, Wang B, Li FH, Tong W (2004) Discovery of immune related factors in *Fenneropenaeus chinensis* by annotation of ESTs. *Prog Nat Sci* 14, 47–54
- Somlyo AP, Wu X, Walker LA, Somlyo AV (1999) Pharmacomechanical coupling: the role of calcium, G-proteins, kinases and phosphatases. *Rev Physiol Biochem Pharmacol* 134, 201–234
- Strom AR, Kaasen I (1993) Trehalose metabolism in *Escherichia coli*: stress protection and stress regulation of gene expression. *Mol Microbiol* 8, 205–210
- Talhok SN, Volkman LE (1991) *Autographa californica* M nuclear polyhedrosis virus and cytochalasin D: antagonists in the regulation of protein synthesis. *Virology* 182, 626–634
- Tsoi SC, Cale JM, Bird IM, Ewart V, Brown LL, Douglas S (2003) Use of human cDNA microarrays for identification of differentially expressed genes in Atlantic salmon liver during *Aeromonas salmonicida* infection. *Mar Biotechnol* 5, 545–554
- van Hulten MC, Witteveldt J, Peters S, Kloosterboer N, Tarchini R, Fiers M, Sandbrink H, Lankhorst R, Vlak JM (2001) The white spot syndrome virus DNA genome sequence. *Virology* 286, 7–22
- van Hulten MC, Reijns M, Vermeesch AM, Zandbergen F, Vlak JM (2002) Identification of VP19 and VP15 of white spot syndrome virus (WSSV) and glycosylation status of the WSSV major structural proteins. *J Gen Virol* 83, 257–265
- Watanabe T, Persson P, Endo H, Kono M (2000) Molecular analysis of two genes, DD9A and B, which are expressed during the postmolt stage in the decapod crustacean *Penaeus japonicus*. *Comp Biochem Physiol B Biochem Mol Biol* 125, 127–136
- Wulfing C, Davis MM (1998) A receptor/cytoskeletal movement triggered by costimulation during T cell activation. *Science* 282, 2266–2269
- Xiang JH (2001) *Disease Occurrence and Control Strategies of Mariculture Organisms* (Beijing: Ocean Press), pp 1–5
- Xiang JH, Wang B, Liu B, Li L, Wang ZZ, Wang XG, Tong W and Li FH (2002) Over 10,000 expressed sequence tags from *Fenneropenaeus chinensis*. In: *Plant, Animal & Microbe Genomes X Conference*, Town & Country Convention Center, San(aq5)
- Yang F, He J, Lin X, Li Q, Pan D, Zhang X, Xu X (2001) Complete genome sequence of the shrimp white spot bacilliform virus. *J Virol* 75, 11811–11820
- Yang GP, Ross DT, Kuang WW, Brown PO, Weigel RJ (1999) Combining SSH and cDNA microarrays for rapid identification of differentially expressed genes. *Nucleic Acids Res* 27, 1517–1523
- Yu CJ, Lin YF, Chiang BL, Chow LP (2003) Proteomics and Immunological Analysis of a Novel Shrimp Allergen, Pen m2. *J Immunol* 170, 445–453