Modulation of Bio-defense Genes in WSSV-Infected *Penaeus monodon*

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ABSTRACT

Penaeus monodon is a host species to white spot syndrome virus (WSSV), the causative pathogen of a serious disease that has impacted the shrimp farming industry all over the world. The present study investigates some of *P. monodon's* cellular defense mechanisms against WSSV in a temporal analysis of 17 genes that are related to the invertebrate immune response. The results suggested that several defense mechanisms were induced upon WSSV infection, including the prophenoloxidase activation system and the JAK/STAT (Janus kinase/Signal Transducer and Activator of Transcription) signal transduction pathway. Conversely, the clotting system and the expression of anti-microbial peptide were down-regulated by WSSV infection.

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INTRODUCTION

The past two decades have shown that the shrimp aquaculture industry is vulnerable to disease, and one of the most economically significant viral pathogens is white spot syndrome virus (WSSV). Outbreaks of WSSV have caused global losses that are estimated at billions of dollars annually (Rosenberry, 2005). Shrimps lack an adaptive immune system of the type found in vertebrates, and even though there is some recent evidence of adaptive or "memory" immunity (Witteveldt *et al.*, 2004a; 2004b), shrimps mostly rely on their innate immune system to defeat infecting pathogens quickly and effectively. The innate immune system in shrimp involves several responses, including hemolymph coagulation, anti-microbial action, free radical formation, phagocytosis, cellular recognition, encapsulation and melanization (Cerenius and Soderhall, 2004; Soderhall and Cerenius, 1998). In addition, certain cellular responses such as apoptosis and heat shock response are also a significant part of the organism's defense against infection. However, these responses damage the host cell as well as the invading pathogen, and regulation of the defense responses therefore needs to be quite sophisticated. For example, in crayfish, the melanization responses are regulated by the prophenoloxidase (proPO) activation system, which is a cascade of serine proteinases that might be further regulated by the Mitogen-Activated Protein Kinase (MAPK) signaling pathway (Mavrouli *et al.*, 2005). In *Drosophila*, the genes encoding anti-microbial peptides are induced in response to microbial infection through the activation of two different but related signaling pathways, Imd and Toll (Engstrom, 1999; Imler and Hoffmann, 2000). In addition to the Imd and Toll pathways, the transcriptional profile of DCV-infected *Drosophila* suggests that the JAK/STAT (Janus kinase/Signal Transducer and Activator of Transcription) pathway might also respond to virus infection (Dostert *et al.*, 2005).

To investigate which immune response pathways might be used by *P. monodon* upon WSSV infection, in this preliminary study, we use RT-PCR to conduct a temporal analysis of 17 genes that are known to be related to immune response in invertebrates. Our results suggest that WSSV infection induces several defense mechanisms, including the proPO activation system and the JAK/STAT signal transduction pathway. Conversely, the clotting system and the expression of anti-microbial peptide are both down-regulated by WSSV infection.

MATERIAL AND METHODS

Virus

The virus used in this study, WSSV Taiwan isolate, originated from a batch of WSSV-infected *Penaeus monodon* shrimp collected in Taiwan in 1994. To prepare the inocula, the epidermis from *P. monodon* with a pathologically confirmed WSSV infection was homogenized (0.1g/ml in 0.9% NaCl) and then centrifuged at 1000Xg for 10 min. The supernatant was diluted to 1:100 with 0.9% NaCl and filtered through a 0.45 um filter.

Shrimp

Adult *P. monodon* (30 to 40 g) were collected from a culture pond at the Biotechnology Division of the Fisheries Research Institute in southern Taiwan. The shrimp used for the challenge test were checked with a commercial diagnostic kit (IQ2000^{TM}, IntelliGene) and confirmed to be WSSV-free. Prior to the experiment, shrimp were kept in a 70 L tank maintained at 25 to 28°C. Experimental shrimp were infected with WSSV by injection using a method described previously (Tsai *et al*., 1999). At various times over the course of the next 24 hrs, two or three specimens were selected at random and their pleopods were immediately frozen and stored in liquid nitrogen until used.

RNA isolation

For each time point, the frozen pleopods from WSSV-infected *P. monodon* were pooled and homogenized in 6 ml Trizol reagent (Life Technologies) and then subjected to ethanol precipitation. The pooled total RNA samples for each time point were then stored in 75% ethanol at -20°C.

Sequences of shrimp bio-defense genes

The sequences of 16 *P. monodon* bio-defense genes were obtained from the nucleotide Expression Sequence Tag (EST) database hosted by NCBI. In addition, the partial sequence of *pmstat* was obtained through RT-PCR amplification with the VNM-STAT-F/R primer set (5'-GAGTCAGTGATGGATGAGAAA-3'/5'-GTCGGAGAAACGGAGCAAGAA-3'), which was designed from a *P. vannamei* EST clone that had a 5' sequence that was similar to STAT. As Table 1 shows, the genes studied here can be categorized into two main groups. The first group includes genes that are involved in innate immunity (Table 1a), and the second group includes genes involved in other putative defense mechanisms (Table 1b).

Temporal analysis of bio-defense genes in *P. monodon* by RT-PCR

Each pooled total RNA sample in 75% ethanol was centrifuged at 14000Xg for 30 min at room temperature. The resulting pellets were re-suspended in DEPC-water and quantified by spectrophotometry at a wavelength of 260 nm. For each time point, an aliquot of 10 ug RNA was treated with 200U of RNase-free DNase I at 37°C for 30 min to remove any residual DNA and then extracted with phenol-chloroform. The DNase–treated total RNA was denatured by heating at 85°C for 10 min in 10 ul DEPC-water containing 100pmol oligo dT primer. The first-strand cDNA was synthesized by the addition of 4 ul SuperscriptII 5X buffer, 1 ul 100 mM DTT, 1 ul 10 mM dNTP, 10 ul RNAsin (Promega), and 100 U SuperscriptII reverse transcriptase (Life Technologies). DEPC-water was added to make a final volume 20 ul. The reverse transcription proceeded at 37°C for 1 hour, followed by heating at 95°C for 5 min to stop the reaction. One tenth of the products of the cDNA were taken to perform RT-PCR in a 50 ul reaction mixture containing 10 mM Tris-Cl, PH 8.8, 1.5 mM $MgCl_2$, 150 mM KCl, 0.1% Triton X-100, 0.2 mM dNTP, 100 pmol of the respective primer sets for each of the tested genes (see Table 1) and 2 U Dynazyme

Table 1. Analyzed bio-defense genes in *Penaeus monodon*.

Involved response	Genes	GenBank accession no.	Primer set	Annealing temp.
a. Genes involved in innate immunity				
Anti-microbial action	anti-microbial peptide	BI018071	5'- TGAGAAGAGCTTTCCTAGTTTAG-3' 5'-TACAAGTTTCTGTTATATTCTCTTG-3'	51
	anti-microbial peptide	BI018074	5'- CAGAAAGGAGTAGTTCATTCCC-3' 5'-TCGTCCGGGTGGTGGTCTAAG-3'	52
	penaeidin-2	BI018089	5'-TGCAGAGGCCGAAACTCCTTG -3' 5'-ACATACATCCCACATGCACTTTC -3'	53
	penaeidin 3-1	BI018080	5'-TCTCACCTGACACTCACCTGC-3' 5'-ACTACAACGAAAGTCAAACAACAC-3'	52
	lysozyme	BI018081	5'-TGGTTGGGCTTCTGGCGTTTC-3' 5'-TGAGATGACATGTATCTGACGTG-3'	53
proPO activation system	hemocyte kazal-type proteinase inhibitor	BI018078	5'-CCGATCATGATTGTATCGGCTAC-3' 5'-TTCCATTTCCTGTCACAAGGTCC-3'	53
	prophenoloxidase	BI018090	5'- TGTGAGGATATATTTGGCTCCGAAG-3' 5'-ATCCCGCAGGAAGGACACACCG -3'	53
	protein C	BI018099	5'-ACAGGTAACCAGATCGCAGGAC-3' 5'-TTGTGGAAATTGGGGTGAATGATG-3'	53
	clip-domain serine protease	BI018087	5'-GACTGCACCTCAACAGGAAGGAC -3' 5'-AATCTGTACCTTCTGCAGCACCTG -3'	54
Clotting reaction	transglutaminase	BI018082	5'-TTTCGGAGGCTGGCAGGTCAT -3' 5'-TAAATACGTATGCAGAATACTGTTC-3'	52
Signaling pathway	protein kinase C inhibitor	BI018088	5'-TGGTGCGCAACATGAGTGACG -3' 5'-ACTGGTGATATGACAAATATGCAG -3'	52
	STAT	this study	5'-GTT CAG TGT GGG AGG TGG AG-3' 5'-AGA AAC GGA GCA AGA AAG TG-3'	52
b. Genes involved in other putative defense mechanisms				
Heat shock response	heat shock protein 10	BI018100	5'-CGGTTCTGCTGCGTTAATCTC-3' 5'-ATGTGATGATTACATACATACATAG-3'	51
	heat shock protein 90	BI018096	5'-ACGAAAGAATAAATCCTTATAACAG -3' 5'-ACACCCGACCATGGCTACATG -3'	51
	heat shock protein 70	BI018094	5'-ACGTGTTACAAGTAGCTCTTAGG -3' 5'-ATACAGTGTTGTTGGGGTTCATC -3'	55
Oxidative stress	peroxidase	BI018092	5'-ACGGAGATCATGGAGTCGTCCCAC -3' 5'-TGCGCCGCCTTCACTTCCTTCATC-3'	55
Others	thymosin beta a	BI018086	5'-TGCGAGACTCACACATTACAATC -3' 5'-TGAGAAGGTCAGCAGGCACTC-3'	53

II DNA polymerase (Finnzyme, Finland). PCR was conducted as follows: 94°C for 2 min, 40 cycles of 94°C for 1 min, annealing at the temperature specified in Table 1 for 1 min, 72°C for 1 min, followed by an elongation at 72°C for 15 min. A beta actin transcript was amplified with the actin-F1/ actin-R1 primer set (5'-GAYGAYATGGAGAAGATCTGG-3' /5'-CCRGGGTACATGGTGGTRCC-3') and used as a reference gene. A WSSV genomic DNA-specific primer set IC-F2/ IC-R3 (5'-CAGACTATTAATGTACAAGTGCG-3'/ 5'-GAATGATTGTTGCTGGTTAGAACC-3') derived from an intergenic region of the WSSV genome was used to confirm that the RNA was not contaminated by any genomic DNA (data not shown).

RESULTS

Transcriptional analysis of bio-defense genes in *P. monodon* after WSSV infection

RT-PCR expression patterns of the pooled pleopod RNA (Figure 1a) suggested that eleven genes were down-regulated after WSSV infection. The proteins encoded by these genes include: five anti-microbial peptides; one protein that is involved in the proPO system; the key enzyme for the clotting reaction; a protein that is involved in anti-oxidative stress; a protein that regulates the Protein Kinase C (PKC) pathway; a protein that is involved in cell shaping and cell migration; and a member of the small heat shock protein family. Among the genes that are up-regulated upon WSSV infection (Figure 1b), two are involved in the proPO system and the third is involved in the JAK/ STAT pathway. The expression levels of two heat shock proteins and another protein involved in the proPO system remained unchanged after WSSV infection (Figure 1c). These data have not been quantified or normalized, but for each time point, the expression level of a beta-actin housekeeping gene was included for comparison (Figure 1d).

DISCUSSION

proPO activation system

One of the most important defense mechanisms, melanization, is mediated by the proPO activation system, and the results (Figure 1) suggest that this system might be activated after WSSV infection. The expression level of both prophenoloxidase and protein C, a non-proteolytic proteinase that can interact with prophenoloxidase activating precursor to enhance the activity of the proPO system (Yu *et al*., 2003), increased significantly from 4 to 6 hrs post-infection. Meanwhile, at about 6 hpi, the expression levels of a kazal family proteinase inhibitor began to decrease. Although these changes suggest that the proPO system may be activated, it should be pointed out that, to date, no direct evidence has been presented to indicate that the kazal-type proteinase inhibitors actually regulate the proPO activation system. It has been shown, however, that inhibitors from the kazal family can inhibit more than one serine proteinase with multiple domains (Kanost, 1999; Kato *et al*., 1987; Reisinger *et al*., 1987). The kazal-type proteinase inhibitor therefore remains a good candidate for controlling proteolytic activity in the proPO activation system.

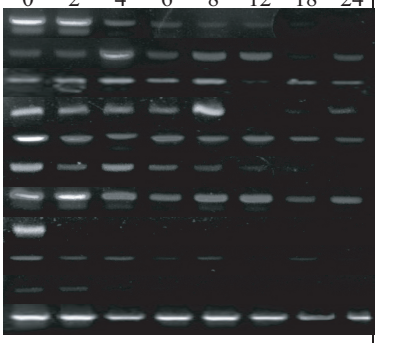
Figure 1. Time-course RT-PCR transcription levels of 17 P. monodon bio-defense genes at 0 to 24 hrs after WSSV infection. Genes are grouped according to their expression patterns. Each time point shows the RT-PCR results for pooled RNA extracted from the pleopods of 2 to 3 experimental infected shrimp.

a. Down-regulated gene expression

Involved response	Genes
Anti-microbial action	anti-microbial peptide
	anti-microbial peptide
	penaeidin2
	penaeidin3-1
	lysozyme
ProPO activation system?	hemocyte kazal-type proteinase inhibitor
Clotting reaction	transglutaminase
Oxidative stress	peroxidase
Signaling pathway	protein kinase C inhibitor
Heat shock reponse	heat shock protein10
Other responses	thymosin beta a

Hpi (hours post WSSV infection)

0 2 4 6 8 12 18 24

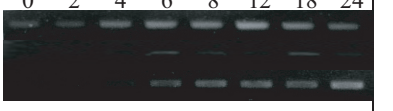


b. Up-regulated gene expression

ProPO activation system	prophenoloxidase
	protein C
Signaling pathway	STAT

Hpi

0 2 4 6 8 12 18 24

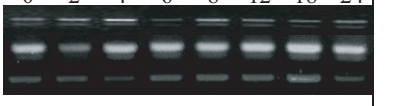


c.Unchanged expression levels

Heat shock reponse	heat shock protein 90
	heat shock protein 70
ProPO activation system	clip-domain serine protease

Hpi

0 2 4 6 8 12 18 24



d. Reference gene

Housekeeping	β - actin

Hpi

0 2 4 6 8 12 18 24

We note too that the proPO activation process produces an abundance of the free radicals that are used to destroy the pathogens (Nappi and Ottaviani, 2000). It may therefore be significant that the expression pattern of peroxidase, an anti-oxidative reagent that protects the cell from damage by free radicals, is down-regulated (Fig. 1a). This may be because the shrimp host is temporarily reducing the expression level of peroxidase to maximize the cytotoxic effect.

JAK/STAT signalling pathway

In vertebrate animals, several cellular responses are mediated by the JAK/STAT signaling pathway, including cell growth, cell differentiation and immune response (Levy and Darnell, 2002). Generally, the activation of STAT requires tyrosine phosphorylation through JAK. This allows the cytoplasmic STAT proteins to form dimers, which then enter the nucleus and bind to the recognition sequences in their respective promoters. However, in addition to JAK, a recent study suggests that inhibition of PKC activity leads to impairment of the tyrosine phosphorylation of STAT that is required for IFN-alpha mediated HCV RNA replicon clearance (Fimia *et al.*, 2004) . Hence, the decreased expression levels of PKC inhibitor (Figure1a) might act to further increase STAT activity by enhancing the activity of PKC.

In insect, the role of STAT appears to be similar. For example, bacterial infection of mosquito induced the expression of STAT, and the activated STAT successively translocated into the nucleus (Barillas-Mury *et al*., 1999) . A recent study in *Drosophila* also suggested that the JAK/STAT signaling pathway is required for the host's antiviral response (Agaisse and Perrimon, 2004; Dostert *et al.*, 2005) . In the present study, *pmstat* was transcriptionally up-regulated after WSSV infection, suggesting that the JAK/STAT signaling pathway might be part of the shrimp antiviral mechanism.

Anti-microbial action

Figure 1a shows that the anti-microbial peptides, which function as detergents to destroy the lipid membrane of pathogens, were down-regulated after WSSV infection, suggesting that anti-microbial actions were weakened by WSSV infection. Although there is no direct evidence, down-regulation of the anti-microbial peptides was presumably mediated by pathways similar to the Toll and Imd pathways in *Drosophila* (Engstrom, 1999; Imler and Hoffmann, 2000).

Clotting reaction

The clotting reaction of hemolymph is another important defense mechanism in invertebrates and it benefits the host by immobilizing the invading pathogens. In shrimp, this system is simple and mainly relies on the enzyme transglutaminase (Huang *et al*., 2004). The down-regulation of the expression of transglutaminase (Fig. 1a) therefore suggests that WSSV weakens the immunity of shrimp by impairing the clotting reaction. This assumption is consistent with the fact that the hemolymph does not coagulate in seriously WSSV- infected shrimps.

Unchanged expression levels

Even when the expression level of a gene is not affected by WSSV infection, the response mediated by that gene may still be important. For example, heat shock protein 90 (Fig. 1c) can be required by both virus and host (Garry *et al*., 1983; Hu *et al.*, 2004; Srivastava, 2002). A constant expression level might therefore simply mean that these genes are already being expressed at levels that are not detrimental to either the virus or the host.

CONCLUSION

In conclusion, although this is only a preliminary study, the transcriptional analysis results suggest that at least four defense mechanisms are modulated by WSSV infection. The proPO activation system and the JAK/STAT signaling pathway are induced after WSSV infection, while anti-microbial action and the hemolymph coagulation mechanism are repressed.

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