Expression analysis of selected haemocyte transcripts from black tiger shrimp infected with yellow head virus

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ABSTRACT: Yellow head virus (YHV) is a lethal Penaeid shrimp virus that has caused significant losses to aquaculture farms raising black tiger shrimp (*Penaeus monodon*). In order to probe some of the underlying molecular events occurring in *P. monodon* cells in response to YHV infection, we investigated the response of 96 selected shrimp haemocyte transcripts from 90 distinct genes at 24 hours post infection using cDNA macroarray hybridization. We found that the transcripts encoding for PMCP423 (innexin-2-like protein) and NF229 (optomotor blind-like protein) showed increased expression, while those encoding for PMC252 (clathrin light chain) and PMC238 (selenoprotein W-like) showed a decreased expression. Interestingly, no significant alteration in expression levels was noted for several transcripts involved in immune or defence functions such as the putative anti-lipopolysaccharide factor, proteinase inhibitor, or crustin-like antibacterial protein. The reduced level of expressed selenoprotein W transcript in YHV-infected shrimp haemocytes might be related to systemic apoptosis and death of the shrimp.

KEYWORDS: yellow head virus, expression, shrimp, haemocyte, transcripts, *Penaeus monodon*

INTRODUCTION

Yellow head virus (YHV) is a lethal shrimp virus that has plagued Penaeid shrimp farms in Thailand and other countries since 1990[1]. One of the susceptible host species is the black tiger shrimp (*Penaeus monodon*), in which the mortality rate in the early years used to reach a level close to 90% within 3–5 days. YHV has been well studied ever since[2,3], and has been found in other Penaeid shrimp species[4]. The YHV genome has been found to be RNA which, together with the viral morphology and partial gene sequences, has led to its classification as a Nidovirus in *Nidovirales*[5,6]. Some genes of the virus, i.e. those of ORF1b, p20 protein and some glycoproteins, have been characterized[7–9]. Early studies of YHV and other shrimp viruses in many laboratories focused on diagnostic detection systems using either PCR or

monoclonal antibodies. These systems are now widely used by the aquaculture industry to screen mother and pre-nauplius shrimp[5,10,11]. A molecular study of YHV infection has led to the identification of a host protein which acts as a viral receptor in lymphoid cells of *P. monodon*[12,13], although other proteins have also been implicated[14]. Studies on *P. monodon* have shown that most cell types are susceptible to the virus, including the primary cell type of the innate defence system, the haemocyte. After infection, the infected cells undergo apoptosis and most of the internal tissues disintegrate[15]. Although RNAi technology has been used to block YHV infection in black tiger shrimp[16,17], the molecular events surrounding the host responses to YHV remain poorly studied.

In contrast to the case of YHV, more molecular studies have been conducted on another lethal shrimp virus, the white spot syndrome virus (WSSV). Based

on either the proteomics approach or microarray approach, a number of proteins or transcripts have been reported to be up or down regulated in both *Penaeus monodon*[18-21] and *Litopenaeus vannamei*[22].

We have previously isolated and sequenced a large number of expressed sequence tags (ESTs) from cDNA libraries of *P. monodon* and several hundred sequences have been deposited in the EST division of GenBank[23]. With the availability of the characterized shrimp cDNA clones in our lab, we were interested to see if the corresponding transcripts might have altered expression upon YHV infection. Healthy black tiger shrimp (free of WSSV and YHV) from aquaculture farms were used as specimens to study the transcriptional response of a selected number of genes by a macroarray-based reverse dot blot hybridization. Twenty-four hours post infection was selected as the time point in this study to obtain a profile of the early transcriptional response of the infected shrimp. Our findings described in this paper provide the first evidence of the importance of these transcripts in the molecular pathogenesis of and/or host responses to YHV infection.

MATERIALS AND METHODS

Original YHV stock was obtained in 2000 from infected juvenile *Penaeus monodon* from a farm in Chachoengsao province near Bangkok. The virus was purified by Urograffin gradient ultracentrifugation as described previously[13], aliquoted to multiple stock tubes, and kept at −80 °C until use. The virus stock was diluted 1:50 with NaCl Tris-EDTA buffer (NTE) prior to use. Black tiger shrimps injected with the diluted virus would normally develop yellow head symptoms and die within 72 h and tested PCR-positive to YHV specific primers.

Live black tiger shrimps were purchased from local vendors and verified to be free from WSSV and YHV by PCR. They were kept in the lab for 1 day to recover from travel and fed only once, but were not fed on the following experimental day. For each experiment, at least 45 shrimps (20–25 g bodyweight) were used and were divided into 3 groups: 0-h control group, 24 h post-buffer injection control group, and 24 h post YHV infection group. Shrimps of the second and the third groups were individually injected with 100 µl of either NTE buffer solution, or the NTE-diluted virus stock. Shrimps in the 0-h control group were not injected and haemolymph was drawn via the sinuses at the base of the walking legs, pooled, and added to cold Alsever's solution at the start of the experiment. Haemolymph from the buffer-injected and virus-infected groups was collected at 24 h post-infection.

To prepare probes, total RNAs were prepared from haemocytes using TRIzol LS reagent (Sigma, St. Louis, MO) as recommended by the manufacturer and dissolved in DEPC-treated water. RNA concentration of each preparation was estimated spectrophotometrically by measuring the absorbance at 260 and 280 nm. An aliquot was removed for RT-PCR analysis of YHV and WSSV status. To generate labelled first strand cDNA probes for use in hybridization reactions, approximately 5 µg of total RNA was incubated with 500 ng of oligo(dT)[13–18] in 12 µl and heated to 65 °C for 5 min before placing samples on ice. Reverse transcription was undertaken in a final volume of 20 µl, containing 1x first strand buffer (GibcoBRL, Gaithersburg, MD), 10 mM DTT, 0.5 mM of dATP, dTTP and dGTP and 50 µCi of $[\alpha\text{-}^{32}P]$ dCTP (Amersham Pharmacia Biotech UK; specific activity 3,000 Ci/mmol) and 200 Units Superscript II reverse transcriptase (GibcoBRL) at a temperature of 42 °C for 50 min. Reaction was terminated by heating to 70 °C for 15 min, and then incubating at 37 °C for 20 min with 2 units of *E. coli* RNaseH (Promega). Unincorporated label was removed by filtration through a Sephadex G-50 column in TE buffer.

A total of 96 cDNAs from 90 distinct genes were selected as hybridization targets in this study. Most clones were isolated from black tiger shrimp haemocyte cDNA libraries prepared in our laboratory (Institute of Molecular Biology and Genetics, Mahidol University, Thailand). The only exception was clone NF229 which was among DNA clones isolated by Thepparit and coworkers from a PCR cloning using degenerate primers[24].

To prepare the target arrays, the insert from each selected cDNA clone was amplified from the parental pBluescript vector used in the original cDNA library preparation by PCR using universal primers (T7 and SK reverse) and confirmed by gel electrophoresis. A total of 100 ng for each insert was denatured by boiling and the sample spotted onto GeneScreen*Plus* membranes. A multiple positive control of *P. monodon* actin (GenBank accession number: AW600674) and a negative control (100 ng of pBluescript) were also included on the membrane. The first round of hybridization was performed on a membrane spotted with the inserts from 96 cDNA clones. A second confirmatory membrane contained a subset of 38 inserts from cDNA clones.

Hybridization was undertaken for 18 h at 65°C in 0.5 M $NaPO_4$ buffer pH 7.0, 7.0% (w/v) SDS, 1mM EDTA pH 8.0, and 1.0 % (w/v) fraction V bovine serum albumin. Following hybridization, membranes

were washed with phosphate-SDS washing solution I (40 mM NaPO$_4$ buffer pH 7.2, 1 mM EDTA pH 8.0, 5.0 % (w/v) SDS, and 0.5 % (w/v) fraction V bovine serum albumin) for 5 min at 65 °C, followed by washing with solution II (40 mM NaPO$_4$ buffer pH 7.2, 1 mM EDTA pH 8.0, and 1.0 % (w/v) SDS) for 5 min at 65 °C three times. Membranes were then exposed to Kodak XAR films at –80 °C overnight. Probes were stripped from the filters by boiling them in a solution of 0.1x SSC, 1.0 % (w/v) SDS for 10 min and verified by Geiger counter and autoradiography.
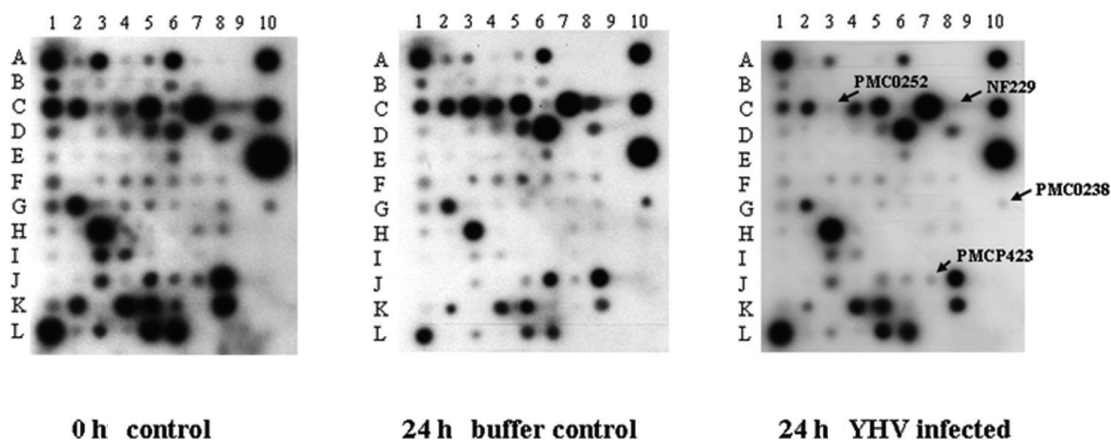
Autoradiographs were scanned using Bio-Rad densitometer GS 700 and signals digitized with Molecular Analyst software version 1.4.1. The signal of each spot was reported as the volume of signal which is equivalent to the mean optical density (background-subtracted) multiplied by the area. The intensity signals among different exposure times of the films was normalized via the hybridized signal of actin positive controls on 3 or 4 corners of each membrane.

RESULTS AND DISCUSSION

Haemocytes are a group of defence cells which play a role in the innate immunity of invertebrates. A number of immune cDNAs have been characterized from shrimp[25,26]. However, it is not known if any of them are involved in defence against virus infections or pathogenesis. In order to survey the molecular events inside the black tiger shrimp haemocytes upon challenging with YHV, macroarray membranes spotted with inserts from cDNA clones of known ESTs

were prepared. ESTs included on the membranes were randomly selected from those characterized in our laboratory. The initial macroarray contained 96 target sequences and was hybridized sequentially with radiolabelled probes prepared from haemocytes from control shrimp (0 h), from mock infected (24 h buffer control), and from shrimp infected with YHV for 24 h (YHV 24 h) (Fig. 1). All signals were also quantified by densitometry. A number of transcripts were expressed at levels too low for detection (Fig.1 row A, spots 7, 8, and 9), while other signals were either at levels only slightly above background, or expressed so strongly that quantification was not possible (Fig. 1, row E, spot 10). However, 44 target spots provided signals that were suitable for analysis. Data were first normalized with respect to actin spots, and then analysed to give a figure of YHV/NTE signal (infected signal over buffer control). A cut-off point of a 50% increase or decrease was selected. Of the 44 targets providing signals suitable for analysis, twenty three showed a greater than 50% change in signal intensity (increase or decrease) while 21 showed no alteration. Examples of targets showing a greater than 50% change include target PMCP423 (position J7), target PMC252 (position C3), target NF229 (position c8), and target PMC238 (position G10).

To confirm the results of the 96 position hybridization, a second smaller macroarray containing 38 cDNA insert targets selected from targets suggested from the first macroarray experiment was constructed. Hybridization was undertaken using probes derived from haemocytes from a new set of shrimp infected identically as to the first group. Fig. 2 shows the

0 h control   **24 h buffer control**   **24 h YHV infected**

Fig. 1 Autoradiographs of a 96 target macroarray. As positive control, non-muscle *P. monodon* actin cDNA was spotted at positions A1, A10, L1. Denatured pBluescript SK(-) plasmid vector was used as a negative control at position L8. Membrane was sequentially hybridized with radiolabelled first strand cDNA transcripts prepared from haemocytes of black tiger shrimp not treated (0 h control), injected with buffer (24 h buffer control) or with YHV (24 h YHV infected). Four targets (with position J7; PMC252, position C3, G10) are arrowed.

Table 1 Quantitative expression of selected transcripts of selected clones.

Spot[a]	Clone ID	Putative Identify	Relative transcription ratio[b] in hybridization experiment	
			I[c]	II[c]
A2	ESTPMCP093	unknown	NA	0.99
A3	ESTPMCP423	innexin-2 like	1.66	1.93*
A4	ESTPMCP259	unknown	0.18	0.73
B2	ESTPMC023	proteinase inhibitor	2.34	1.17
C1	ESTPMC286	CG6891-like protein	4.72	0.97
C2	ESTPMCP119	antibacterial protein	NA	1.03
D1	ESTPMC284	L7 ribosomal protein	4.38	0.86
D2	ESTPMCP111	S9 ribosomal protein	2.65	1.03
E2	ESTPMC28	actin	1.60	1.03
E4	ESTPMC0238	selenoprotein W	0.27	0.03*
F2	ESTPMC417	antibacterial protein	1.56	1.07
F3	ESTPMC252	clathrin light chain	0.09	0.27*
F5	ESTPMCP126	proteinase inhibitor	0.49	0.75
G1	ESTPMC0401	ADP/ATP Translocase	4.98	1.39
G2	ESTPMC0220	elongation factor 1	1.12	0.54
G3	NF229	optomoter blind like	HBg	2.59*
H1	ESTPMCP276	antilipopolysaccharide factor	1.93	1.00
I2	ESTPMC0298	S2 ribosomal protein	1.14	0.60
I4	ESTPMCP069	innexin 2	NA	0.98
A1,A5, I1,I5	ESTPMC0011	actin (from shrimp haemocytes)	positive control	

[a] Spot positions are reported according to experiment shown in Fig. 2

[b] Expression ratio was calculated with respect to buffer injection control

[c] Experiments I and II used different batches of shrimp

* Targets with markedly altered expression ratio

HBg = High background (not determined). NA = Not available. Positive control spots (for detection and exposure normalization) are A1, A5, I1, I 5. Negative control spot was H5 (pBluescriptSK(-) plasmid vector). No sample was spotted in positions D3, E3.
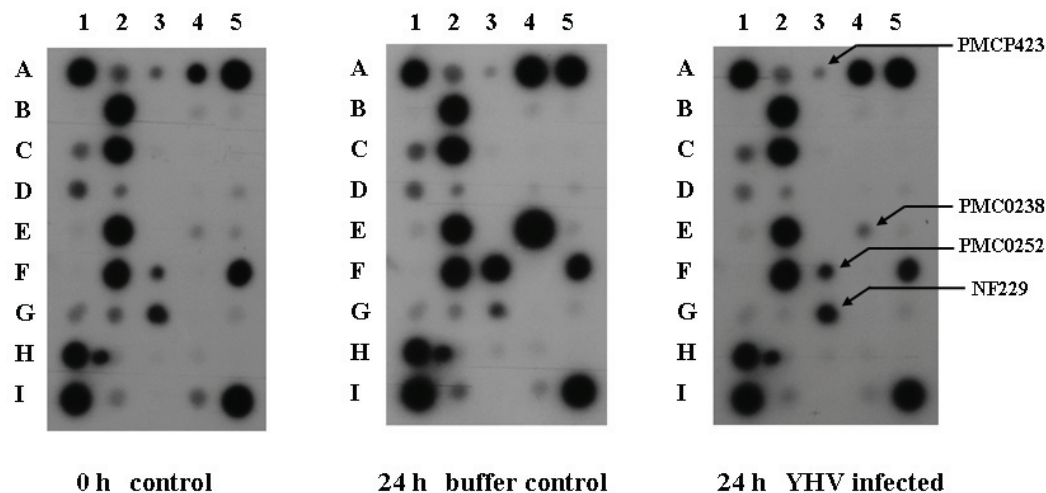
three autoradiographs derived from this experiment. Autoradiographs were normalized and analysed for the 96 target macroarray and results of selected spots are given in Table 1. Two targets, ESTPMCP423 (for innexin-2 like protein) and NF229 (for a transcription factor, optomotor blind-like protein), showed an increase in expression of greater than 50%, while two targets, ESTPMC252 (for clathrin light chain) and ESTPMC238 (for selenoprotein W), showed a decrease in expression of greater than 50%. Several targets showed variations that were slightly below the 50% threshold. For example, ESTPMC401 (for ADP/ATP translocase) showed a slight increase in expression on the second macroarray (1.39×) but a significantly higher increase in expression in the first macroarray (4.98×), suggesting that slight variations in the batches of shrimp may produce slightly different results. It should be noted that ESTPMCP069, another putative Innexin-2, was not responsive. Full length nucleotide sequences of the two shrimp innexin-2 clones revealed an overlap of 220 bases, with the YHV-responsive ESTPMC423 clone having a longer 3′ end (data not shown). It is reasonable to conclude that the observed increased transcription signal for ESTPMC423 is likely to be due to the effects of the probe sequences at the 3′ end.

Our work is among the first to address the question of the transcriptional response of haemocytes to YHV infection. Our results indicate that levels of haemocyte transcripts of a gap junction protein innexin-2 like protein (ESTPMC423) and a transcription factor called optomotor blind like protein (NF229) increase after YHV infection of black tiger shrimp. Increased transcripts for the innexin-like protein might indicate an increased porosity of the haemocytes' cell membrane.

Interestingly, some cDNA classified as involved in bacterial defence or immune functions did not show an altered response to YHV infection. Included in the macroarray were two cDNA clone inserts (ESTPM-CP119 and ESTPMC417) for the antibacterial peptide (crustin-like). DNA sequences of the two clones are almost identical, sharing an overall 97% sequence identity (data from one-pass DNA sequencing) and it is possible that the two ESTs arose from transcripts of the same gene. Neither gene, however, showed any transcriptional response to YHV infection (1.03× and 1.07×, compared to buffer control). Other shrimp cDNA clones implicated in the pathogenic response system that did not show a transcriptional response

Fig. 2 Autoradiographs of a 38 target macroarray. As a positive control, non-muscle *P. monodon* actin cDNA was spotted at positions A1, A5, I1, and I5. Denatured pBluescript SK (-) plasmid vector was used as a negative control at position H5. Membrane was sequentially hybridized with radiolabelled first-strand cDNA transcripts prepared from haemocytes of black tiger shrimp not treated (0-h control), injected with buffer (24 h buffer control) or with YHV (24 h YHV infected). Four targets are arrowed.

at 24 h post infection include antilipopolysaccharide factor (1.00×, position H1) and proteinase inhibitor (1.17× position B2 and 0.75×, position F5).

The transcription level of transcripts for the clathrin light chain and selenoprotein W decreased in response to YHV infection. A reduction in the level of clathrin light chain would serve to reduce endocytotic activity. This could be a molecular sign of immunologically impaired haemocytes in infected shrimp. In *Drosophila*, selenoprotein W has been shown to be involved in the protection against oxidative stress and apoptosis[27], suggesting that the reduced selenoprotein W transcript seen in the YHV-infected haemocytes might be an early signal for turning on the apoptotic response. This is in line with a known phenomenon that YHV infected shrimp cells undergo apoptosis[15]. It has been shown that the massive induction of apoptosis in YHV infected shrimp haemocytes can reduce the levels of actin[28]. Studies in WSSV-infected *Penaeus monodon*, however, provided conflicting results. Whereas Wongpanya et al[20] reported an increased in transcript levels of beta actin and actin 1 upon WSSV infection, Wang et al[21] reported a decreased level of actin protein. However, we believe the use of actin as a marker for early transcriptional events is still valid as there was not a loss of bulk haemocytes within this time frame.

In this paper, we have described some of the molecular events occurring in haemocytes of the black tiger shrimp in response to infection with YHV. Our findings should pave the way for further studies to elucidate the mechanism of YHV's pathogenicity and the death of host cells. We also envisage that some of the cDNAs which were found to have an altered transcription level due to YHV infection might be useful as molecular probes to check on the health status of shrimps in aquaculture ponds or of their cultured cells.

ACKNOWLEDGEMENTS

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