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*Original Article*

# Detection of *PinX1* and *14-3-3* in the shrimp (*Litopenaeus vannamei*) and study on gene expressions during viral infection and environmental stresses

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**Abstract**

Two genes, *PinX1* and *14-3-3,* have been isolated and investigated for their expression in shrimp, *Litopenaeus vannamei* when infected with white spot syndrome virus (WSSV) and subjected to environmental stresses. A putative PinX1 protein of 180 amino acids showed a 65% similarity to the zebra fish PinX1 protein (*Danio rerio*) and had a G-patch domain similar to human PinX1. The sequence of a full length cDNA of *14-3-3* has a very high similarity (96%) to other shrimp 14-3-3-like protein (*Feneropenaeus merguiensis* and *Penaeus monodon*). Transcripts of *PinX1* and *14-3-3* were up regulated in the hemolymph of viral infected shrimp with the highest expression level at 24 hrs p.i. Shrimp showing mortality characteristics had very low expression of these two genes. In animals subjected to a combined low temperature (19-20°C) and low oxygen (DO 1-1.5 mg/L) for 24 hrs, an interesting result was that the transcript of *PinX1* was drastically increased. In contrast, *14-3-3* did not show any significant differences between the six treatments. The results of this work indicated that the PinX1 protein might play an important role in the shrimp response to viral infection and repose to certain stresses. In contrast the 14-3-3 protein might play a particularly important role in the immune defended mechanisms of viral infections of shrimps.

**Keywords:** *Litopenaeus vannamei*, WSSV, stress, PinX1, 14-3-3

## 1. Introduction

White spot syndrome virus (WSSV) has been the most serious viral disease of farmed penaeid shrimp. WSSV can cause up to 100% shrimp mortalities within 2-10 days and also infects other species of aquatic organisms, including crabs and crayfish (Lo *et al.*, 1996; Chen *et al.*, 1997; Flegel, 1997). It has been suggested that apoptosis induced by WSSV may be a part of the pathophysiology that leads to shrimp death (Flegel and Pasharawipas, 1998; Flegel, 2001). This does occur in *Penaeus monodon* infected with yellow-head virus (Khanobdee *et al.*, 2002) and WSSV (Wongteera-supaya *et al.*, 1995). Within two years after a WSSV disease outbreak, shrimp develop some kind of adaptive tolerance (immunity) to the disease whenever subsequently challenged with the same virus. The survivors became infected but showed no gross signs of the disease. This phenomenon now known as the “viral accommodation concept” was first described by Flegel in 1998 and there have been subsequent updates (Flegel and Pasharawipas, 1998; Flegel, 2007). It is thought that biomolecules, produced by the shrimp, restrict the action of the viral trigger for apoptosis and allow the shrimp to survive. It is important to identify these biomole-cules.

In previous work, several genes that are involved in the shrimp cell death pathway were isolated from cDNA

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subtractive and ESTs libraries of shrimp infected with WSSV (Bangrak *et al.*, 2002; Bangrak *et al.*, 2004; Tassanakajon *et al.*, 2006). Among them, a translationally controlled tumor protein (*TCTP*) or *Fortilin* gene has been intensively studied. This gene is down-regulated in moribund *P. monodon* infected with WSSV. A fortilin recombinant protein did protect cells from death when cells were incubated under toxic conditions. This indicates that it can function as an anti-apoptotic protein and may play a critical role in the shrimp response to WSSV infection. *Caspase-3*, a key enzyme in the apoptosis pathway was recently cloned and characterized (Phongdara *et al.*, 2006; Senapin *et al.*, 2007). Caspase-3 expression in *F. merguiensis* was assayed using the standard kits designed for detecting mammalian caspase. This indicates that the caspase gene may have been well conserved during evolution. As a result of this finding we have predicted that other molecules in the shrimp cell death pathway may be evolutionarily conserved.

In this work we cloned and characterized another two genes obtained from subtractive cDNA and ESTs libraries. Their sequences showed statistically significant similarities to the human homologues of *PinX1* and *14-3-3*. Apart from these two genes that produced putative proteins involved in the apoptosis pathway, a heat shock protein, *Hsp70* was also included in this study. Hsp 70 proteins have been shown to play a critical role in cell survival and thermotolerance in response to environmental stresses (Parsell and Lindquist, 1993; Parsell *et al.*, 1993; Lo *et al.*, 2004). A stress response is a ubiquitous phenomenon in all organisms and can be considered as a general cellular reaction to physiological disturbances. For aquaculture, environmental stresses such as temperature, hypoxia/anoxia and salinity, may be commonly faced by many organisms in the aquatic environment (Zenteno-Savin *et al.*, 2006) and effect all biological processes from molecules to behavior. Signs of stress can be overt, such as sluggishness, lack of feeding activity, slow growth, molting difficulties, hyperactivity, death, or hiding until animals become ill. The action of stress on shrimp is varied and not widely studied. One consequence of the viral accommodation concept is the proposal that the maintenance of virus in the host population is mainly by vertical transmission of very low viral doses from broodstock to larvae such that there are no disease symptoms in the absence of appropriate stress triggers. Therefore the stress trigger might be another important factor associated with disease outbreak. It was therefore of interest to compare the response of the four target proteins listed above to viral infection and environmental stress. How these proteins respond to the daily stresses caused by pathogens and environmental changes may ultimately determine the health of the shrimp.

## 2. Materials and Methods

### 2.1 Animals

White shrimp (*Litopenaeus vannamei*) were obtained from Aquatic Animal Research Health Center, Department of Aquatic Science, Prince of Songkla University, Satun Province, Thailand. The shrimp were reared in three tons, fiber tank fitted with aeration and filled with seawater at 27.0 ±1, 30±1 ppt salinity. Shrimp were acclimatized in the fiber tanks for at least two weeks and were fed with commercial diets. Only apparently healthy shrimp at the intermolt stage weighing 8-10 g were used for the study.

### 2.2 WSSV experimental infection

Adult shrimp (*L. vannamei*) weighing about 10 g were kept for two weeks in a 60 l aquarium for acclimatization before experiments were started. Hemolymph (200-250 µl) withdrawn from individual shrimp before the experiment was labeled as 'normal hemolymph'. Shrimp were divided into two groups; a WSSV infected group and a control group. Shrimp were inoculated intramuscularly with 100 µl of a $1:10^6$ dilution of the WSSV stock solution for the WSSV infected group and 100 µl of 1xPBS for the control group. After injection, hemolymph was withdrawn using a 26 ga/l inch needle at 3, 12, 24, 48, and 72 hrs and when moribund.

### 2.3 Experimental design for environmental stress treatment

The shrimp were sampled randomly from the holding tank and transferred individually to 200 l plastic tanks containing 100 L of aqueous test solution. There were six treatments (Table 1), temperature: 19-20°C, 26-28°C and 32-34°C; dissolved oxygen: 1.0-1.5 ppm and 6.0-7.0 ppm. The control group was maintained at a temperature of 26-28°C and dissolved oxygen of 6.0-7.0 ppm. During the experiment temperature and dissolved oxygen (DO) were monitored every 2 hrs and each tank was covered with a plastic cap to prevent the shrimp from escaping. For each treatment the shrimp were exposed to the test stress conditions for 24 hrs and collected for analysis at 0, 3, and 24 hrs.

### 2.4 RNA isolation

Total RNA was prepared from tissue using Trizol LS reagent (GIBCO BRL life Technology). Briefly, after with-

Table 1. Experimental design for environmental stress treatment

| Treatment | Salinity (ppt) | Dissolved oxygen (mg/L) | Temperature (°C) |
|---|---|---|---|
| 1 | 30±1 | 6.0-7.0 | 26-28 |
| 2 | 30±1 | 6.0-7.0 | 32-34 |
| 3 | 30±1 | 6.0-7.0 | 19-20 |
| 4 | 30±1 | 1.0-1.5 | 26-28 |
| 5 | 30±1 | 1.0-1.5 | 32-34 |
| 6 | 30±1 | 1.0-1.5 | 19-20 |

drawing the hemolymph, 200 µl was vigorously mixed in 200 µl of Trizol LS reagent by vortex. Then, 0.1 ml of chloroform was added and vigorously shaken. The sample was centrifuged at 12,000 rpm for 15 min. Subsequently, the aqueous phase was removed, transferred to a fresh tube and mixed with 0.25 ml of isopropyl alcohol to precipitate RNA. The sample was incubated at 30°C for 20 min and then centrifuged at 12,000 rpm for RNA precipitation. These total RNA pellets were dried in a vacuum, re–suspended in 50 µl of RNase free water and were stored at -70°C prior to use. The amount of the RNA was determined by spectrophotometry at 260 nm.

### 2.5 Cloning of *PinX1* and *14-3-3* cDNA from *L. vannamei*

The partial sequences of *PinX1* and *14-3-3* were amplified by RT-PCR using primers designed, base on DNA sequences from subtractive cDNA library (for *PinX1*) and ESTs library (for *14-3-3*) (Bangrak *et al.*, 2002; Tassanna-kajon et al., 2006). All PCR products were cloned into pGEM-TEasy (Promega Corporation) and sequence analysis using the ABI prism 377 DNA Sequencer (Perkin-Elmer, Norwalk, CT, USA). Gene database searches were performed through the BLAST network server at http://www.ncbi.nim.nih.gov (National Center for Biotechnological Information Bethesda, MD, USA). The sequence alignments were created using the Clustal X program.

### 2.6 Analysis of gene expression of *PinX1* and *14-3-3* during viral infection and environmental stress

To determine the expression of *PinX1*, *14-3-3, Fortilin* and *Hsp 70* during viral infection and environmental stress, RT-PCR analysis and semi-quantitative assay were performed. Primers designed for each of the detected sequences and synthesized by Life Technologies, USA are shown in Table 2. For viral infection experiment, total RNA of various tissues of the normal and WSSV-infected shrimp, and the hemolymph of WSSV-induced shrimp at various time were investigated. For the environmental stress experiment, six treatments were prepared as described above and analyzed. After total RNA quantification, about 1 mg of the DNase I treated total RNAs from each sample was used as the template in 50 ml of the RT-PCR reaction mixture according to the manufacturer's instructions (Access RT-PCR System and Access RT-PCR Instructory System, Promega). For all amplifications, the reaction was started at 48°C for 45 min followed by an initial PCR activation step at 94°C for 2 min followed by 30 cycles of 94°C for 30 sec, 52°C for 1 min and 68 °C for 2 min. Elongation factor and b-actin genes were used as an internal control in semi-quantitative analysis and to ensure that the RT-PCR for each sample contained the same amount of total RNA and that the RNA was intact. The ratio of interested gene to housekeeping gene was calculated from the image using Scion Image software. Semi-quantitation by RT-PCR was carried out in triplicate.

### 2.7 Statistical analysis

Statistical significance was calculated using one-way ANOVA followed by a Tukey test. Results with a $p<0.05$ were considered significant for the mRNA expression experiments.

## 3. Results

### 3.1 Isolation of *PinX1* and *14-3-3* from *L. vannamei*

Sequencing of partial *PinX1* (Genbank accession number EF408930) and *14-3-*3 (Genbank accession number EF408929) cDNAs from *L. vannamei* obtained by RT-PCR showed a 540 bp and 324 bp encoding the polypeptide consisting of 180 and 108 amino acid residues, respectively. The BLASTX search showed that the encoded protein of *L. vannamei*-PinX1 showed 65% similarity to the PinX1 protein of zebra fish, *Danio rerio*, whereas the deduced amino acid sequence of *L. vannamei*-14-3-3 had the highest similarity to other shrimp 14-3-3-like proteins from *Fenneropenaeus*

Table 2. Oligonucleotide primers used for cloning and expression analysis.

| Primer | Sequence (5′→3′) | PCR fragment size (bp) | Tm (°C) |
|---|---|---|---|
| *EF*-Forward<br>*EF*-Reverse | GTCTTCCCCTTCAGGACGTC<br>GTCGATCTTGGTCAGCAGTTC | 390 | 5149 |
| *Hsp 70*-Forward<br>*Hsp 70*-Reverse | ATGGCAAAGGCACCTGCTGTCGGT<br>ACTTCTCCACGGGCTCCAAGGTG | 958 | 5656 |
| *PinX1*-Forward<br>*PinX1*-Reverse | ATGTCTATGTTGGCGGAACCAAGG<br>CACCATTCAACTTCACCTGCTGAC | 551 | 5252 |
| *14-3-3*-Forward<br>*14-3-3*-Reverse | ATGTCGGACAAGGAAGAACAAGTAC<br>GCTTTGGGAATAAGGAACTTGTCG | 329 | 5151 |
| *Fortilin*-Forward<br>*Fortilin*-Reverse | CTCACCAGTCGAGAATTTAGCGACG<br>CTGGCCGCTCTTCTCCATCAATGTC | 502 | 5555 |

*merguiensis* and *Penaeus monodon* at 96%. The *L. vannamei-PinX1* cDNA had a 5' G-patch domain (28-70 aa) containing six highly conserved glycine residues that have been proposed to mediate RNA binding (Aravind and Koonin, 1999). The multiple sequence alignments of the deduced amino acid sequences of PinX1 and 14-3-3 with other known PinX1 and 14-3-3 orthologs are shown in Figure 1 and 2, respectively.

### 3.2 Expression of genes in response to WSSV infection

The response of Fortilin after viral infection has been reported previously (Bangrak *et al.*, 2004; Graidist *et al.*, 2006). Here, we focused on the genes encoding *PinX1*, *14-3-3* and *Hsp70* by using RT-PCR analysis of total RNA, extracted from the hemolymph of normal, PBS injected, WSSV injected and moribund shrimp. Primer for *Hsp70* was from DNA sequences of *L. vannamei – Hsp70*. The results (Figure 3) demonstrated that there was a low expression of *PinX1* and *14-3-3* in PBS injected shrimp. *PinX1* however was highly expressed at 24 hrs and so was *14-3-3* at 12, 24, and 48 hrs post injection with WSSV. There was a very low level of expression of both genes when shrimp began showing the characteristics of mortality (Figure 3). In contrast the expression of *Hsp70* was the same for all samples tested.

### 3.3 Expression of genes in response to environmental stress

To investigate whether the expression profile of the genes encoding *PinX1*, *14-3-3*, *Fortilin* and *Hsp 70* were influenced by temperature and DO stresses, we performed RT-PCR analyses assays using specific primers. The gene expressions were analyzed in three individuals of each group of samples. *PinX1*, *14-3-3*, and *Fortilin* showed more expression at 24 hrs than at 3 hrs in all stress treatments (Figure 4A-C). In contrast *HSP 70* expression increased at 3 hrs and reduced at 24 hrs after being subjected to any of the stresses, but especially in treatment 5 (high temperature/low DO) (Figure 4D). *Fortilin* was highly expressed at either high or low temperature with low DO. *14-3-3* did not show any significant differences between the 6 treatments. *PinX* was similar to *Fortilin*, in that it was highly expressed in low DO; however the effects was more prominent with combined low temperature and low DO.

### 3.4 Tissue distribution of *PinX1* and *14-3-3*

The tissue distribution of *PinX1* and *14-3-3* transcripts was investigated in the following organs: stomach, gill, hepatopancreas, muscle, lymphoid organ, nerve, heart and intestine. Small amounts of *PinX1* transcripts were

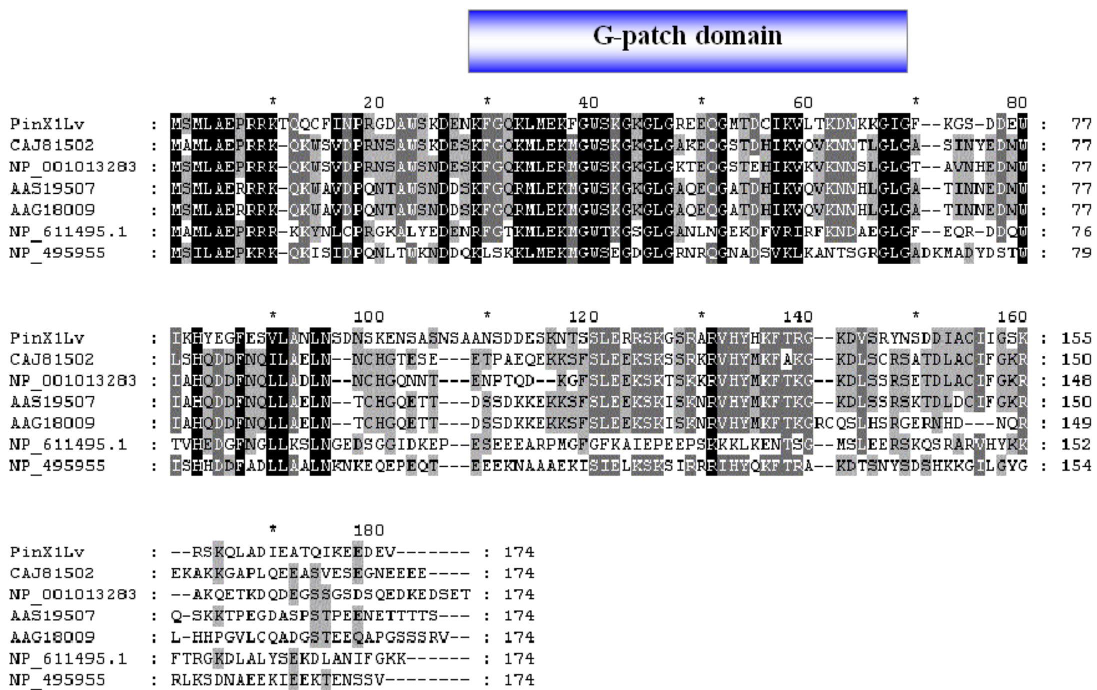


Figure 1. Multiple alignment of the amino acid sequences of *Litopenaeus vannamei -PinX1* (PinX1Lv) with that of various PinX1s; CAJ81502 (*Xenopus tropicalis*), NP_001013283 (*Danio rerio*), AAS19507 (*Homo sapiens*), AAG18009 (LPTS *Homo sapiens*), NP_611495.1 (*Drosophila melanogaster*) and NP_495955 (*Caenorhabditis elegans*). The dash represents an amino acid gap at the indicated position in the protein. Positions where the amino acid residue is conserved in 100, 80, and 60% of sequences are highlighted in black, dark grey and light grey, respectively. A bar represents the G-patch domain.

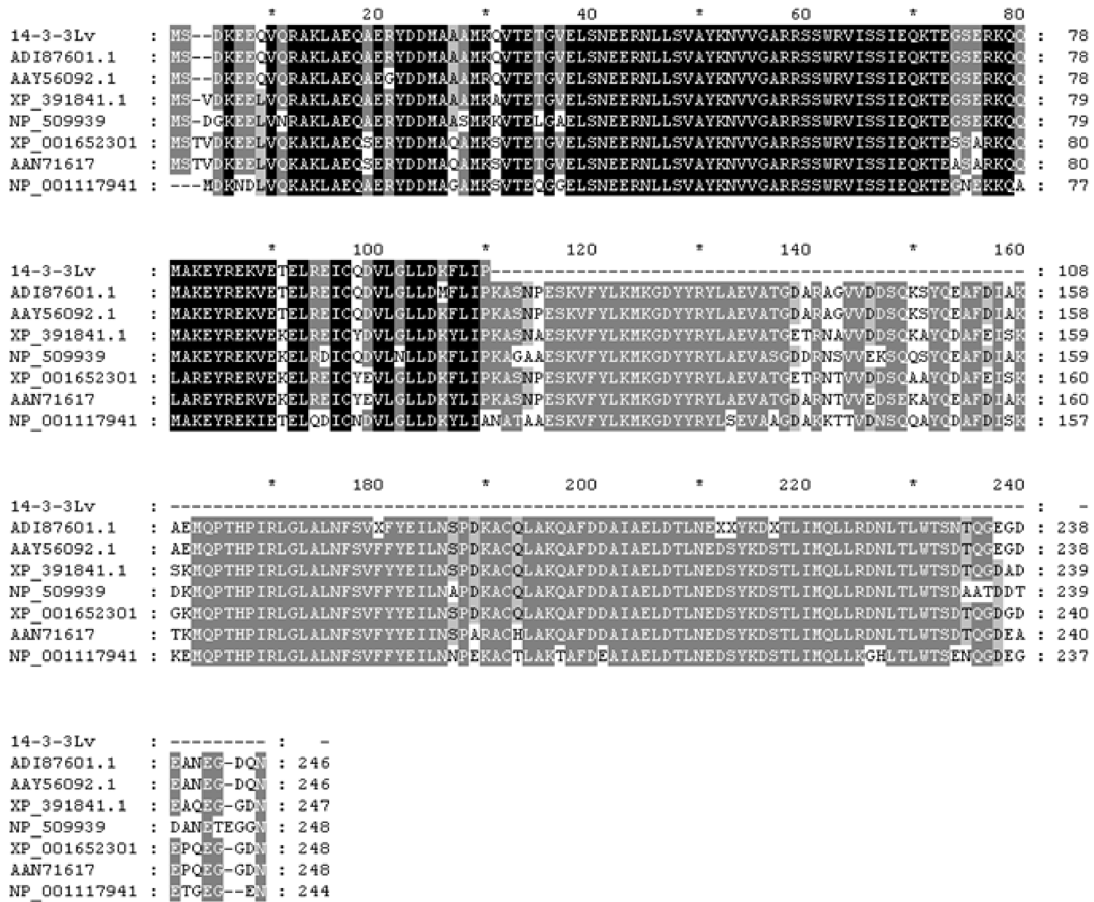


Figure 2. Multiple alignment of the amino acid sequences of *Litopenaeus vannamei* -14-3-3 (14-3-3Lv) with that of 14-3-3 proteins from other species; ADI87601.1 (*Fenneropenaeus merguiensis*), AAY56092.1 (*Penaeus monodon*), XP_391841.1 (*Apis mellifera*), NP_509939 (*Caenorhabditis elegans*), XP_001652301 (*Aedes aegypti*), AAN71617 (*Drosophila melanogaster*) and NP_ 001117941.1 (*Oncorhynchus mykiss*). The dash represents a gap in the amino acid at the indicated position in the protein. Positions where the amino acid residue is conserved in 100, 80, and 60% of sequences are highlighted in black, dark grey and light grey, respectively.

detected in all normal tissues. *14-3-3* transcripts were detected in moderate amounts in all tissues. In the viral infected experiment, the expression of *PinX1* was significantly increased in stomach, gill, muscle, nerve, heart and intestine (Figure 5). Whereas the expression of 14-3-3 was significantly increased in two organs tested, including hepatopancreas and heart.

## 4. Discussion

The rapid global increase in the cultivation of penaeid shrimp has unfortunately resulted in massive farm losses mostly due to viral pathogens. In the past decade, research has concentrated on a closer examination of the shrimp response to viral pathogens in the hope of finding new methods to control the spread of diseases. In 2007, an update of the 'viral accommodation concept' by Flegel (2007) stated "*crustaceans and other arthropods actively accommodate viral pathogens as persistent infections so that they act as a kind of memory that functions to specifically reduce the severity of disease and to dampen viral triggered apoptosis*." A number of publications on shrimp viral infections have tested this concept. An increased level of apoptosis in moribund shrimp leading to mortality was reported in *L. vannamei* and *P.* monodon infected with White Spot Syndrome Virus (WSSV) (Sahtout *et al.*, 2001; Wongprasert *et al.*, 2003) and Yellow Head Virus (YHV). In *P. japonicus*, it was found that the level of apoptosis was high in moribund shrimp susceptible to WSSV but low in those that survived WSSV challenge due to a ''quasi immune'' protection (Wu and Muroga, 2004). In order to obtain a better understanding of the interaction of virus and host in viral accommodation, more detailed molecular studies are required. Our work has focused on the biomolecules that participate in the apoptosis pathway. Several genes that are involved in the shrimp immune response, signal transduction and apoptosis pathways have been collectively isolated and characterized (Sri-

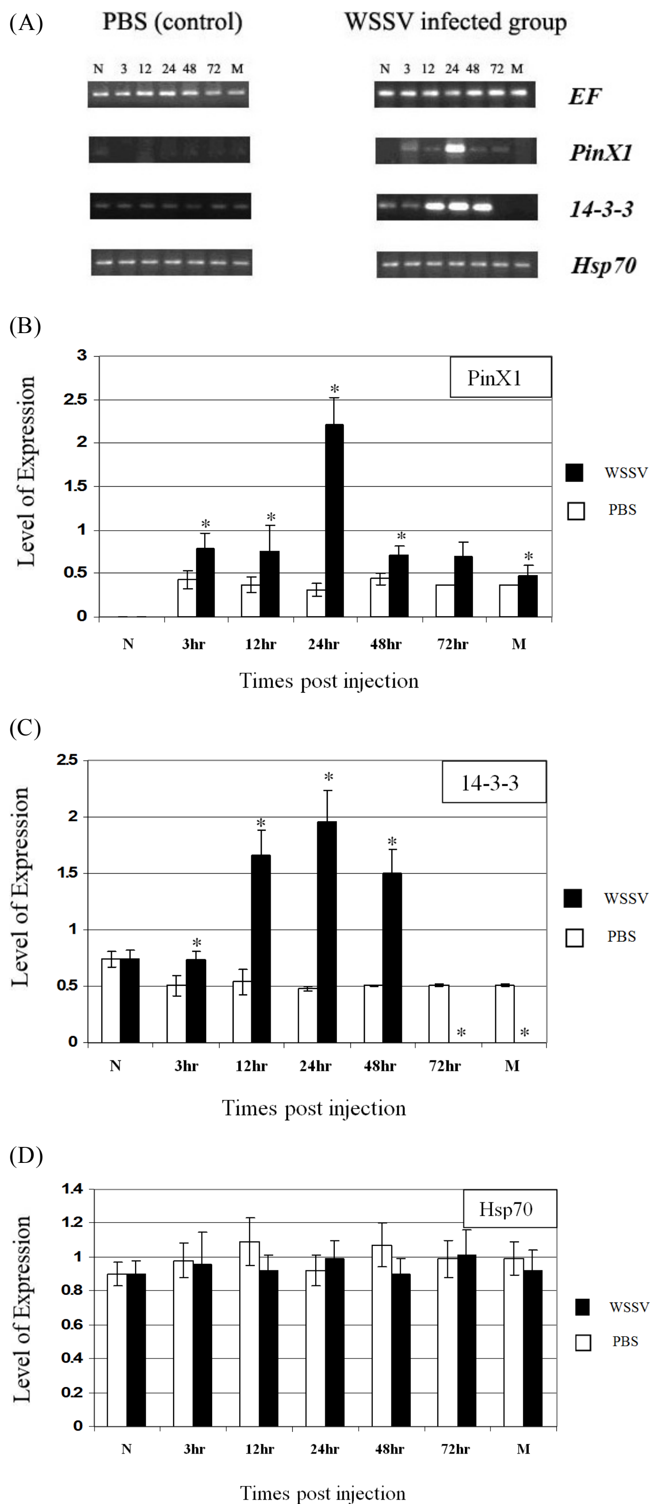


Figure 3. (A) RT-PCR assay of *PinX1*, *14-3-3* and *Hsp* gene expression in hemocytes of experimentally infected shrimp at various time post challenge (3, 12, 24, 48, 72 hrs post-injection; M= moribund infected shrimp; N = normal shrimp). The expression is compared to elongation factor (EF) as an internal control. (B) Normalized *PinX1*, (C) *14-3-3* and (D) *Hsp* expression were calculated from the images using Scion Images software. The significant difference ($p<0.05$) indicated in asterisk (*).

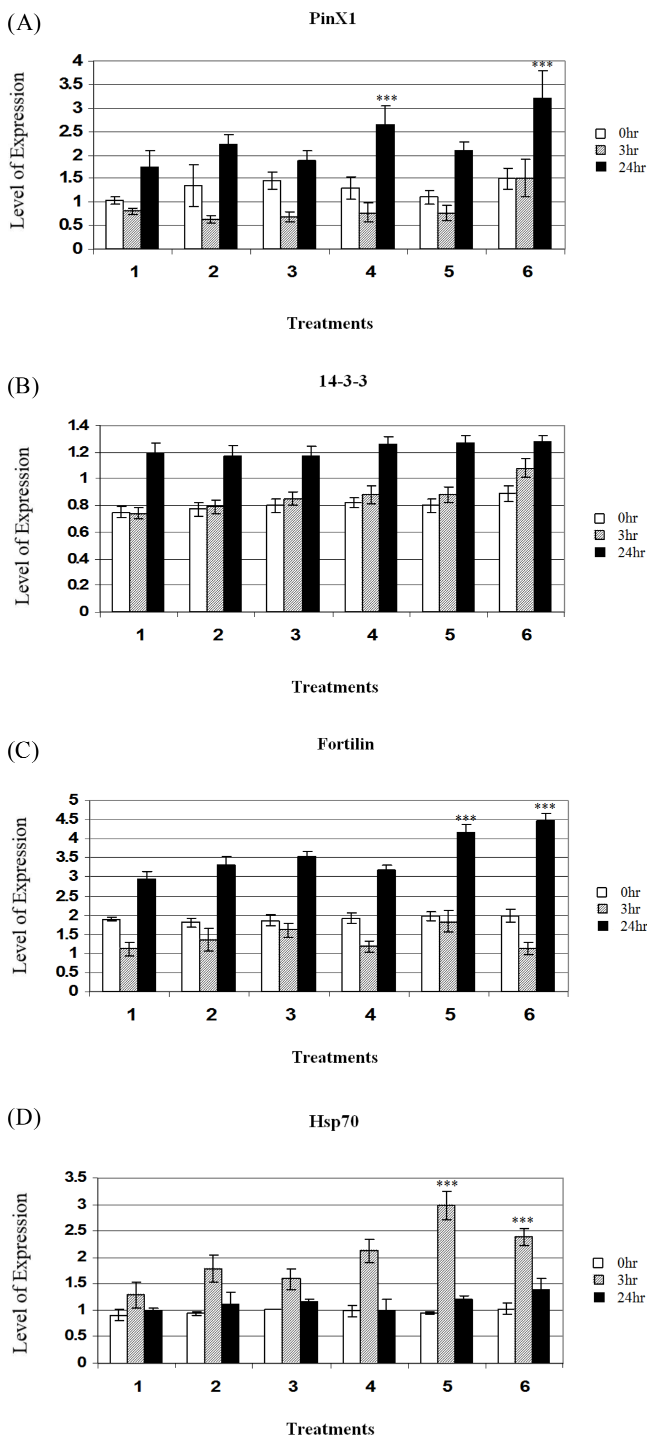


Figure 4. Semi-quantitative gene expression analysis of, (A) *PinX1*, (B) *14-3-3*, (C) *Fortilin* and (D) *Hsp70* in response to temperature and DO stresses at 0, 3 and 24 hrs. The ratio of gene expression to EF was calculated from the image using Scion Image software. ***, $p<0.0001$ in comparison of treatment 2-6 with treatment 1.
Treatment 1, Temp. 26-28°C, DO 6-7 mg/L; Treatment 2, Temp. 32-34°C, DO 6-7 mg/L; Treatment 3, Temp. 19-20°C, DO 6-7 mg/L; Treatment 4, Temp. 26-28°C, DO 1-1.5 mg/L; Treatment 5, Temp. 32-34°C, DO 1-1.5 mg/L; Treatment 6, Temp. 19-20°C, DO 1-1.5 mg/L.

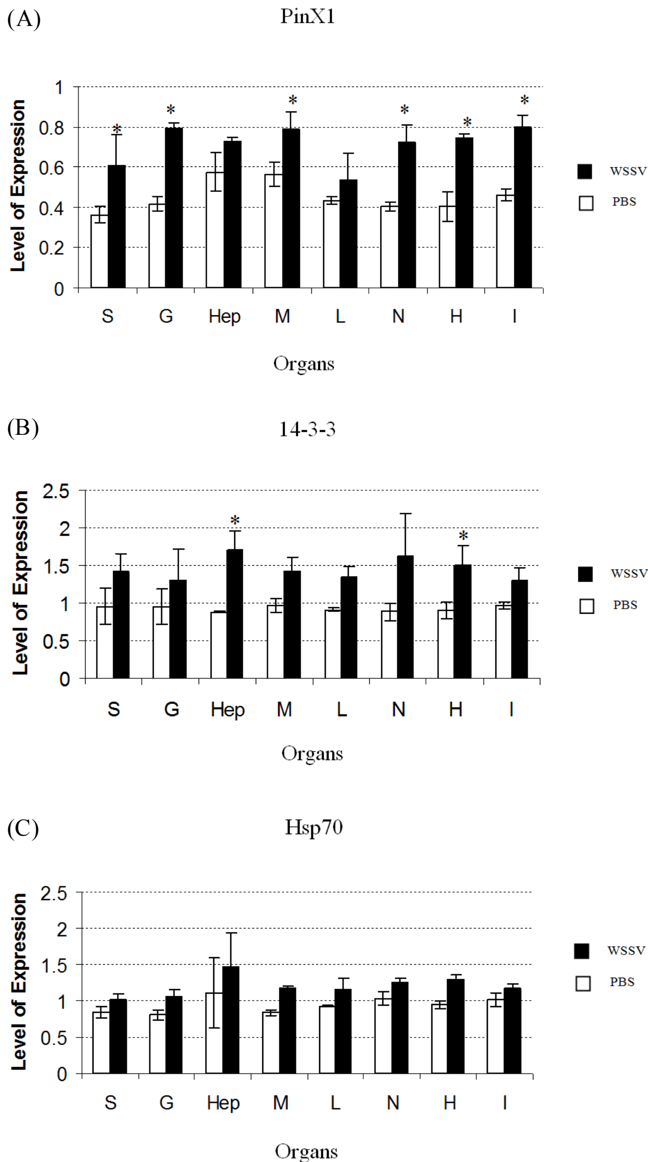


Figure 5. PCR analysis of specific transcripts of *PinX1* (A), *14-3-3* (B) and *HSP70* (C) in various tissues; 1) stomach, 2) gill, 3) hepatopancreas, 4) muscle, 5) lymphoid organ, 6) nerve, 7) heart, 8) intestine. Tissues were obtained from normal and WSSV infected shrimp. The PCR product of EF was used as an internal marker. The ratio of gene expression to EF was calculated from the image using Scion Image software. The significant difference ($p<0.05$) indicated in asterisk (*).

tunyalucksana *et al.*, 1999; Bangrak *et al.*, 2002; Bangrak *et al.*, 2004; Tonganunt *et al.*, 2005). The recent discovery that expression of the anti-apoptotic translationally controlled tumor protein (TCTP) or *Fortilin* gene is down-regulated in moribund *P. monodon* infected with WSSV seems to support the viral accommodation concept. Here we isolate and characterize another two genes, *PinX1* and *14-3-3*.

The human homologue of PinX1 is a potent telomerase inhibitor and a putative tumor suppressor (Zhou and Lu, 2001). It is a conserved protein and PinX1 ORF's are found in other eukaryotic cells. Human *PinX1* encodes a novel 328 aa protein containing no known unique domain structure except an N-terminal Gly-rich patch (Aravind and Koonin, 1999). The shrimp *PinX1* cDNA encodes a 180 amino acid protein and contains a 5' G-patch domain (Figure 1). G-patches are short conserved sequences of about 48 amino acids containing six highly conserved glycine residues that have been proposed to mediate RNA binding (Aravind and Koonin, 1999) and have been found in tumor suppressors and DNA-repair proteins (Pang *et al.*, 1993; Zhou and Lu, 2001; Dendouga *et al.*, 2002; Chaouki and Salz, 2006; Frenal *et al.*, 2006). The highest similarity of our clone is 65% to zebra fish (NP_001013283). The obtained sequence is much shorter than that expected from the human *PinX1* homologue. Unfortunately, the putative sequence of the 3'end was not recovered in this work, although several attempts were made to isolate the full-length cDNA. It is possible that the shrimp PinX1 is a smaller protein similar to the putative liver tumor suppressor gene, 8p23, near to the microsatellite marker D8S277 (Liao *et al.*, 2000). This small 174 amino acid protein has almost identical sequences to 113 residues of human PinX1 and shrimp PinX1. RT-PCR analysis has also shown that the 8p23 transcript is down regulated or absent in about 42% of hepatocellular carcinoma cell lines. The possibility that the shrimp PinX1-like protein is a smaller molecule compared to its human homologue will remain an open question and further investigations are required.

The serine/threonine binding proteins, 14-3-3, constitute a family of eukaryotic proteins that are key regulators of a large number of processes ranging from mitosis to apoptosis. This protein functions as a dimer and binds to particular motifs in their target proteins (Rosenquist *et al.*, 2001). A 14-3-3 protein is involved in the regulation of apoptosis through multiple interactions with proteins of the core mitochondrial machinery, pro-apoptotic transcription factors, and their upstream signaling pathways. Importantly, 14-3-3 plays a critical role in mediating survival kinase-induced signaling to suppress apoptosis (Rosenquist, 2003) and is also a substrate for *Caspase-3* (Won *et al.*, 2003).

Semi-quantitative RT-PCR analysis of both PinX1 and 14-3-3 has shown that their transcripts are up-regulated after viral infection with the highest expression level at 24 h (p.i.) and being down-regulated when the shrimp becomes moribund. This result is very similar to the gene expression profiles of other anti-apoptosis genes, such as *Fortilin* that has been reported earlier by us, and implies that they might participate in the same pathway (Bangrak *et al.*, 2002; Bangrak *et al.*, 2004; Tonganunt *et al.*, 2005). When compared to the well known heat shock protein Hsp 70, the gene that is expressed upon environmental stress (Lo *et al.*, 1004), expression of this gene did not significantly change during the time course of the viral infection experiment.

Interestingly, when the expression level of these genes has been followed in samples obtained from shrimp stressed

by environmental conditions, it was found that after animals were subjected to a combined low temperature (19°C - 20°C) and low oxygen (DO 1-1.5) for 24 hrs, the transcript of *PinX1* was drastically increased. A similar result was also observed but to a lesser extent with *Fortilin*. Graeber *et al.* (1994) present evidence that short-term exposure to hypoxia causes the accumulation of the tumor suppressor p53. However the increase in p53 levels in response to hypoxia is not due to a general stress induced increase in protein synthesis, since hypoxia caused a reduction in overall cellular protein synthesis.

The results of this work indicate that the *PinX1* protein has a particularly important role in the shrimp response to viral infection and response to certain stresses. It would be interesting to determine if the shrimp PinX1 does function like the other G-patch containing proteins in RNA processing or tumor suppressors or DNA-repair proteins and 14-3-3 as an anti-apoptotic protein. The cloning and characterization of these two genes is of benefit for future investigations on cell death and the viral accommodation survival pathway in shrimp.

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