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Short communication

Differential gene expression profile from haematopoietic tissue stem cells of red claw crayfish, *Cherax quadricarinatus*, in response to WSSV infection

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ABSTRACT

White spot syndrome virus (WSSV) is one of the most important viral pathogens in crustaceans. During WSSV infection, multiple cell signaling cascades are activated, leading to the generation of antiviral molecules and initiation of programmed cell death of the virus infected cells. To gain novel insight into cell signaling mechanisms employed in WSSV infection, we have used suppression subtractive hybridization (SSH) to elucidate the cellular response to WSSV challenge at the gene level in red claw crayfish haematopoietic tissue (Hpt) stem cell cultures. Red claw crayfish Hpt cells were infected with WSSV for 1 h (L1 library) and 12 h (L12 library), respectively, after which the cell RNA was prepared for SSH using uninfected cells as drivers. By screening the L1 and L12 forward libraries, we have isolated the differentially expressed genes of crayfish Hpt cells upon WSSV infection. Among these genes, the level of many key molecules showed clearly up-regulated expression, including the genes involved in immune responses, cytoskeletal system, signal transduction molecules, stress, metabolism and homestasis related genes, and unknown genes in both L1 and L12 libraries. Importantly, of the 2123 clones screened, 176 novel genes were found the first time to be up-regulated in WSSV infection in crustaceans. To further confirm the up-regulation of differentially expressed genes, the semi-quantitative RT-PCR were performed to test twenty randomly selected genes, in which eight of the selected genes exhibited clear up-regulation upon WSSV infection in red claw crayfish Hpt cells, including DNA helicase B-like, multiprotein bridging factor 1, apoptosis-linked gene 2 and an unknown gene-L1635 from L1 library; coatomer gamma subunit, gabarap protein gene, tripartite motif-containing 32 and an unknown gene-L12-254 from L2 library, respectively. Taken together, as well as in immune and stress responses are regulated during WSSV infection of crayfish Hpt cells, our results also light the significance of cytoskeletal system, signal transduction and other unknown genes in the regulation of antiviral signals during WSSV infection.

1. Introduction

White spot syndrome virus (WSSV), as one of the most serious pathogens for shrimp farming at present, causes up to 100% mortality within 7–10 days in commercial shrimp farms (Liu et al., 2009). This virus has a wide host range of crustaceans, including shrimps, crab, lobster, and crayfish. It has been well acknowledged that crustaceans rely solely on innate immune response which has a critical role in monitoring virus infection and mounting antiviral responses. Recently, different techniques have been applied to host-WSSV interactions to identify immune factors linked to viral infection in crustaceans (Lan et al., 2006; Liu et al., 2006; Wang et al., 2006, 2007; Zhao et al., 2007). Genes activated by WSSV infections and genes whose expression is associated with the ability of host to survive from viral infections have been widely described (Liu et al., 2009). For instance, virus-inhibiting proteins or genes could be produced and some genes were up-regulated towards viral infection in crustaceans (Dhar et al., 2003; He et al., 2005; Pan et al., 2005, 2000; Rojtinnakorn et al., 2002; Roux et al., 2002). These molecules were shown to be associated with an antiviral response or the immune defense process of viral infection such as the antiviral genes including ALF (Liu et al., 2006; Tharntada et al., 2009), ALDH and HSP70 (Lin et al., 2011), *Lv*CTL1 (Zhao et al., 2009), penaeidin5 (Woramongkolchai et al., 2011), *Plg*C1qR (Watthanasurorot et al., 2010), *Pm*AV (Luo et al., 2003), *Pm*-fortilin (Nupan et al., 2011), synthetin-like protein (Bangrak et al., 2002; Tonganunt et al., 2005) and *Lv*TRAF6 (Wang et al., 2011).

It is well known that hemocyte, one of the most important innate immune effector cell in crustaceans, plays a key role in antiviral defense by phagocytosing virus (Wu et al., 2008) and releasing antiviral molecules (Liu et al., 2006). It can detect microbial infection by recognition of invading pathogens. Furthermore, hemocyte also acts in another crucial antiviral defense-apoptosis, a programmed cell death, which is induced in the viral infected

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cells by yet undefined signaling process to restrict viral infection (Liu et al., 2009). In freshwater crayfish, the matured hemocyte is originated from the haematopoietic tissue (Hpt) (Söderhäll et al., 2003) located at the back dorsal side of its stomach. And differentially expressed genes have been identified from the freshwater crayfish, *Pacifastacus leniusculus*, hemocyte (Liu et al., 2006). However, the molecular mechanisms that underlie the anti-WSSV immune reactions are still unclear due to a lack of cell line for WSSV replication. It has been described that the freshwater crayfish Hpt is a target by WSSV and the Hpt cell culture is suitable for WSSV replication (Jiravanichpaisal et al., 2006). Additionally, several functional studies of anti-WSSV genes have been carried out in this useful cell culture model (Liu et al., 2006; Tharntada et al., 2009; Watthanasurorot et al., 2010). These studies together suggest that crayfish Hpt cell cultures will benefit the mechanism study of anti-WSSV infection at the cellular level.

In preliminary experiments, we found WSSV is able to replicate in the Hpt cell cultures from the red claw crayfish-*Cherax quadricarinatus*, which has also been proved to be infected by WSSV in the animals (Shi et al., 2000). Hence, this cell culture could be useful for characterizing the signal pathway recruited in both the viral invasion and the antiviral process. In order to identify antiviral molecules in the Hpt cell culture of the *C. quadricarinatus*, suppression subtractive hybridization (SSH) was used to compare differentially expressed genes in the Hpt cells of the red claw crayfish between pre- and post-WSSV challenges, since the Hpt cell is important as progenitor of hemocyte that, plays a crucial role in host defense against pathogen invasion in crustaceans.

2. Materials and methods

2.1. Animals, Hpt cell culture and WSSV infection

Freshwater crayfish, *C. quadricarinatus*, purchased from Zhangpu, Fujian, China, were maintained in aerated tap water at 20 °C. The Hpt cells were isolated from the normal inter-molting red claw crayfish, *C. quadricarinatus*, as described by Söderhäll et al. (2003). Briefly, the haematopoietic tissue was dissected from the dorsal side of the stomach and washed with CPBS (crayfish phosphate buffer saline: 10 mM Na_2HPO_4; 10 mM KH_2PO_4; 150 mM NaCl; 10 μM $CaCl_2$ and 10 μM $MnCl_2$; pH 6.8) and then incubated in 500 μl of 0.1% collagenase (typeI and IV) (Sigma, Steinheim, Germany) in CPBS at room temperature for 45 min to dissociate the Hpt cells. The separated cells were washed twice with CPBS by spinning down at 2500 × *g* for 5 min at room temperature. The cell pellet was then re-suspended in modified L-15m81 medium (Söderhäll et al., 2005) and subsequently seeded at a density of 2×10^5 cells/500 μl in 24-well plates. Hpt cells were supplemented with a crude astakine preparation (Söderhäll et al., 2005) after about 30 min attachment at 25 °C.

WSSV was kindly provided by Prof. Xun Xu (Third Institute of Oceanography, SOA, Xiamen, Fujian, China). The virus was prepared as described by Xie et al. and quantified by absolute quantification of PCR (Xie et al., 2005). For viral infection experiments, 10^6 copy of virons/well was inoculated into the 1 day old Hpt cell cultures and incubated for 40 min for viral attachment at 25 °C. Thereafter, the cells were washed twice with L-15m81 medium and cultured with 500 μl of L-15m81 medium together with 5 μl of crude astakine preparation. The cell cultures were then kept for different time periods (0, 1, 3, 6, 12 and 24 h) at 25 °C followed by total RNA preparation.

Total RNA was extracted using RNAprep pure Micro Kit (Tiangen, Beijing, China) with DNase I digestion, and 500 ng of total RNA was prepared for cDNA synthesis with PrimeScript™ RT reagent Kit (Takara). To determine the WSSV infection conditions, IE1 (forward GGTATTGAGGTGATGAAGAGGCG; reverse TGACATGGGAACCACTGTTGAG) and VP28 (forward GGGAACATTCAAGGTGTGGA; reverse GGTGAAGGAGGAGGTGTTGG) genes were employed for RT-PCR detection. Red claw crayfish GAPDH gene (forward AATGCTTCTTGCACCACCAAC; reverse AGGTCTTGCTCAGCTGGATACC) (GenBank No. AY430092.1) (Dhar et al., 2009) was recruited as an internal control. The PCR was prepared by using an equal amount of cDNA (2 μl of 10 times diluted cDNA/reaction) using HS™ Kit (Dongsheng Biotech, China) with the following conditions: 94 °C for 2 min; 32 cycles for GAPDH gene, 40 cycles for IE1 and 35 cycles for VP28 with 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s; a final extension for 2 min at 72 °C. The PCR products were then subjected to 2% agarose gel electrophoresis followed by ethidium bromide staining and visualized under ultraviolet light.

2.2. Construction and screening of subtracted cDNA libraries

The viral infection assay was performed as described above and the infection time was established for 1 h and 12 h, respectively, according to the time-setup mentioned above. Total RNAs from post-WSSV-infection and pre-WSSV-infection were used as the "tester" and "driver". Two forward SSH libraries were prepared as L1 (1 h post viral infection) and L12 (12 h post viral infection), respectively. The library constructions were performed using the Clontech PCR-select™ cDNA Subtraction kit (Clontech). The subtracted target cDNAs were subsequently cloned into a pMD18-T vector (Takara) and transformed into DH5 α Competent Cells (Tiangen, Beijing, China). Recombinant white colonies were randomly picked out and amplified by bacterial-PCR (bacterial clony as PCR template) using a primer pair corresponding to the pMD18-T vector sequences. The PCR products were visualized on 1% agarose gels to ensure quality and quantity of amplification. The PCR products were then spotted onto a positive-charge nylon membrane and cross-linked by a UV cross-linking (Hoefer UV-crosslinker, Amersham, U.S.A.) for further dot-blot screening. Two identical blots were prepared for hybridization with different probes. The total secondary PCR products from the forward and reverse SSH were used as probes. Probes were labeled with digoxigenin-dUTP (Roche, Mannheim, Germany). The blots were pre-hybridized in DIG Easy Hyb solution at 37 °C for 30 min. The probes, produced by PCR using SMART cDNA as template and target specific primers, were labeled by random primed technique with Digoxigenin-11-dUTP according to the manufacturer's instruction. After hybridization at 37 °C overnight, the membrane was washed twice in 2 × SSC–0.1% SDS at room temperature and twice in 0.5 × SSC–0.1% SDS at 65 °C. Blots were auto-radiographed with empirically optimized exposure times. Differentially expressed clones were prepared for sequencing (Invitrogen, China). Sequences were analyzed by NCBI BLAST search program.

Semi-quantitative RT-PCR was adopted to compare the differential expression of responsive genes in pre-WSSV and post-WSSV (1 h and 12 h, respectively) infected Hpt cell cultures. Semi-quantitative RT-PCR was performed as previously described (Pan et al., 2005). Briefly, total RNA was prepared from the Hpt cell cultures. Single-stranded cDNA was synthesized as described above. The PCR was performed for optimized cycles with gene-specific primers and 2 μl of 10× diluted cDNA as template. PCR products were analyzed on 2% agarose gel. To normalize the gene expression data between pre- and post-WSSV infection in crayfish Hpt cell cultures, red claw crayfish GAPDH gene was employed as a suitable reference control gene against WSSV infection (Dhar et al., 2009).

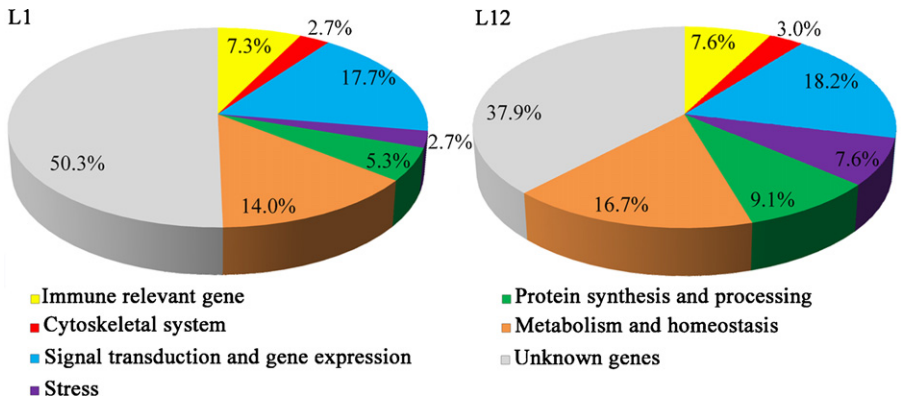

Fig. 1. Distribution of the L1 and L12 isolated transcripts based on database comparison. The genes were classified into seven groups which include genes coding for proteins involved in immune reaction, cytoskeletal system, signal transduction and gene expression, stress, protein synthesis and processing, metabolism and homeostasis, and unknown genes.

3. Results

3.1. Differential screening and analysis of SSH libraries from crayfish Hpt cell cultures post WSSV infection

Although the mechanism of WSSV infection has been the focus of intensive investigation by many researchers, the precise cellular events that mediate WSSV invasion have not been fully elucidated. During viral infection, the host cell activates multiple signaling pathways that result in the antiviral responses. To elucidate the molecular responses to WSSV in red claw crayfish Hpt cell cultures, we first cultured Hpt cells (Fig. S1) and determined the time-course for WSSV infection. As shown in Fig. S2 the transcripts of IE1, an immediate early gene of WSSV, could be observed within 1 h post WSSV attachment (i.e. 40 min after virus innoculation) followed by an increased expression till 24 h in the current study. Additionally, a late gene of VP28, one of the viral structural component genes, showed expression within12 h and remained at high level till 24 h post WSSV infection. We then performed SSH to identify WSSV target genes in crayfish Hpt cells at two different time points as: the crayfish Hpt cells were infected with WSSV for 1 h and 12 h, respectively, to screen the early regulated genes and the late regulated genes of host-cell involved in WSSV infection.

Next, differentially expressed genes between 1 h (L1 library, 1478 clones) and non-viral infection cells, or 12 h (L2 library, 645 clones) and non-viral infection cells were isolated. To further screen the up-regulation of differentially expressed genes, dot-blot hybridization was employed to reveal the genes preliminarily screened by subtractive hybridization. Of the 1478 randomly picked clones, 596 clones were defined as differentially expressed genes by dot-blot screening in L1 library. For L12 library, 208 clones showed up-regulation from 645 randomly selected clones. The different clones with clearly enhanced expression were picked out for sequencing determination followed by NCBI-Blast analysis. WSSV sequences were eliminated and rare sequences were retained by screening the SSH libraries. Comparison analysis of L1 library revealed 300 genes among 447 clones tested were clearly up-regulated (Table 1). In addition, 66 genes among 131 clones exhibited obvious up-regulation in the L12 library (Table 2).

The WSSV associated up-regulated genes encode proteins including a wide range of functional categories in both L1 and L12 libraries. In L1 library, 49.7% of the total genes (i.e. 149 genes) have high similarity with known functional genes, including genes coding for proteins involved in immune reactions (7.3%), cytoskeletal system (2.7%), signal transduction and gene expression (17.7%), stress related genes (2.7%), protein synthesis and processing (5.3%) and metabolism and homeostasis (14.0%). Notably, is that 50.3% had unknown functions (Fig. 1). In L12 library, 62.1% of the total genes (i.e. 41 genes) exhibit high similarity with known functional genes, including genes coding for proteins involved in immune reactions (7.6%), cytoskeletal system (3.0%), signal transduction and gene expression (18.2%), stress related genes (7.6%), protein synthesis and processing (9.1%), metabolism and homeostasis (16.7%) and unknown genes (37.9%) (Fig. 1). In total, 176 novel genes (151 genes from L1 and 25 genes from L12, respectively) were found for the first time to be involved in response to WSSV infection in crustaceans.

3.2. Conformation of up-regulated genes from SSH libraries by semi-quantitative RT-PCR

To further confirm the up-regulation of differentially expressed genes in L1 and L12 libraries, we examined the transcripts of twenty randomly selected genes by semi-quantitative RT-PCR from the subtracted libraries. Crayfish Hpt cells were infected with WSSV for different time periods and total RNA was prepared. As shown in Fig. 2, transcripts of eight selected genes were shown to be highly enhanced by WSSV challenge. WSSV infection had an obviously increased expression of DNA helicase B-like gene (DHB), multiprotein bridging factor 1 (MBF1), apoptosis-linked gene 2 (ALG-2) and L1-635 (an unknown gene) in L1 library. The other genes respectively encoding Serine/Threonine protein kinase (FIKK) and TAR RNA-binding protein 2 (TARRB) from L1 exhibited a lower up-regulation after WSSV infection (data not shown). For L12 library, the transcripts of coatomer gamma subunit (Coa-γ), gabarap protein gene (gabarap), tripartite motif-containing 32 (TRIM32) and

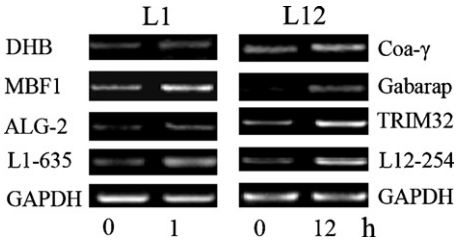

Fig. 2. Confirmation of up-regulated expressed genes by semi-quantitative RT-PCR. The viral infection and cDNA synthesis were performed as described in Section 2. Randomly selected genes from L1 and L12 libraries were determined for semi-quantitation post WSSV infection by RT-PCR. Red claw crayfish GAPDH gene was employed as an internal control. DHB: DNA helicase B-like; MBF1: multiprotein bridging factor 1; ALG-2: apoptosis-linked gene 2; Coa-γ: coatomer gamma subunit; Gabarap: gabarap protein gene; TRIM32: tripartite motif-containing 32; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Table 1
Differentially expressed genes from L1 library after WSSV challenge.

Genes	Accession no. of L1 genes	Related species	E-value	Clones
1. Immune relevant gene				22
Anti-lipopolysaccharide factor isoform 6	JF284545	*Penaeus monodon*	6.00E-07	1
Apoptosis-linked gene 2	JF284559	*Strongylocentrotus purpuratus*	4.00E-27	1
Ferritin 3	JF284490	*Eriocheir sinensis*	2.00E-28	2
Hemocytin	JF284466	*Camponotus floridanus*	2.00E-31	1
Kazal-type proteinase inhibitor	JF284535	*Pacifastacus leniusculus*	9.00E-22	1
Laminin receptor	JF284519	*Litopenaeus vannamei*	6.00E-32	1
α2 Macroglobulin isoform 2	JF284493	*Fenneropenaeus chinensis*	2.00E-51	1
Myelin expression factor 2	JF284604	*Camponotus floridanus*	3.00E-55	1
PAPII	JF284533	*Pacifastacus leniusculus*	3.00E-46	1
Probable Bax inhibitor-1	JF284530	*Osmerus mordax*	7.00E-08	1
Protein späetzle	JF284468	*Camponotus floridanus*	8.00E-07	1
Serine proteinase inhibitor 8	JF284541	*Penaeus monodon*	2.00E-28	3
Serine proteinase-like 2b	JF284531	*Pacifastacus leniusculus*	6.00E-86	1
TAR RNA-binding protein 2	JF284492	*Fenneropenaeus chinensis*	6.00E-55	1
T-complex protein 1	JF284476	*Culex quinquefasciatus*	3.00E-45	4
Transglutaminase	JF284534	*Pacifastacus leniusculus*	4.00E-37	1
2. Cytoskeletal system				8
β Actin	JF284489	*Eriocheir sinensis*	6.00E-79	1
Clathrin light chain	JF284465	*Camponotus floridanus*	1.00E-38	1
Dynein light chain roadblock-type 2	JF284507	*Harpegnathos saltator*	2.00E-18	1
Fimbrin/plastin	JF284598	*Acyrthosiphon pisum*	1.00E-30	1
Tubulin related gene	JF284537	*Paracentrotus lividus*	2.00E-70	4
3. Signal transduction and gene expression				53
Barrier-to-autointegration factor	JF284459	*Caligus rogercresseyi*	1.00E-22	1
Casein kinase 2, alpha 1 polypeptide	JF284550	*Saccoglossus kowalevskii*	7.00E-44	1
CDC5 cell division cycle 5-like	JF284555	*Salmo salar*	5.00E-10	1
CHK1 checkpoint-like protein	JF284509	*Helicoverpa armigera*	6.00E-15	1
DNA helicaseB-like	JF284564	*Xenopus* (*Silurana*) *tropicalis*	7.00E-16	1
Double-strand-break repair protein rad21-like protein	JF297946	*Harpegnathos saltator*	9.00E-22	1
Double-stranded RNA-specific editase Adar	JF284469	*Camponotus floridanus*	1.00E-06	1
Dystroglycan	JF284501	*Harpegnathos saltator*	3.00E-48	1
Elongation factor 1	JF284520	*Litopenaeus vannamei*	1.00E-13	2
Endonuclease and reverse transcriptase-like protein	JF284457	*Bombyx mori*	1.00E-31	1
Histone family	JF284499	*Harpegnathos saltator*	2.00E-67	2
Hnrnp protein	JF284560	*Tribolium castaneum*	7.00E-51	1
Insulin-like growth factor binding protein 7 related gene	JF284483	*Danio rerio*	5.00E-08	2
Kelch domain containing 2-like	JF284551	*Saccoglossus kowalevskii*	1.00E-18	1
Lysyl-tRNA synthetase	JF284474	*Culex quinquefasciatus*	2.00E-10	1
Beta-NAC-like protein	JF284549	*Reticulitermes flavipes*	6.00E-14	1
Neuronal membrane glycoprotein M6-b	JF284500	*Harpegnathos saltator*	1.00E-50	1
Nucleolar protein 1, 120 kda	JF284482	*Danio rerio*	3.00E-28	1
Nuclear import 7 homolog	JF284473	*Ciona intestinalis*	3.00E-38	1
Ras-related GTPase	JF284496	*Glossina morsitans morsitans*	1.00E-42	1
Recombination repair protein	JF284485	*Drosophila melanogaster*	6.00E-11	1
Regulation of nuclear pre-mRNA domain-containing protein 2	JF284511	*Homo sapiens*	3.00E-11	1
Replication factor C subunit	JF284538	*Pediculus humanus corporis*	5.00E-41	1
RNA-binding protein	JF284467	*Camponotus floridanus*	5.00E-24	1
SAPK substrate protein 1	JF284504	*Harpegnathos saltator*	8.00E-25	1
Serine/threonine protein kinase, FIKK family	JF284546	*Plasmodium falciparum*	1.00E-26	1
Signal peptidase complex, subunit SPC25	JF284515	*Ixodes scapularis*	2.00E-26	1
Signal sequence receptor beta-like protein	JF284498	*Haliotis discus discus*	6.00E-34	1
Small nuclear ribonucleoprotein polypeptide G	JF284542	*Penaeus monodon*	2.00E-25	1
Ras-related protein Rap-lb precursor	JF284557	*Scylla paramamosain*	8.00E-88	1
Splicing factor	JF284524	*Nasonia vitripennis*	7.00E-14	3
Structural maintenance of chromosomes smc	JF284599	*Acyrthosiphon pisum*	1.00E-30	2
SWI/SNF complex subunit SMARCC2	JF284512	*Ixodes scapularis*	4.00E-85	1
Tetraspanin-11	JF284484	*Danio rerio*	8.00E-07	1
Transcriptional adapter 1	JF284548	*Rattus norvegicus*	9.00E-18	1
Transcription initiation factor IIF, small subunit	JF284514	*Ixodes scapularis*	1.00E-33	1
Transmembrane emp24 domain-containing protein 10 precursor	JF284527	*Oncorhynchus mykiss*	9.00E-06	1
Transmembrane protein 184B	JF284502	*Harpegnathos saltator*	2.00E-42	1
U3 small nucleolar RNA-associated protein 18 homolog	JF284554	*Salmo salar*	3.00E-28	1

Table 1 (*Continued*)

Genes	Accession no. of L1 genes	Related species	E-value	Clones
Vascular endothelial growth factor A precursor, putative	JF284540	*Pediculus humanus corporis*	1.00E-06	1
WD-repeat protein	JF284513	*Ixodes scapularis*	6.00E-45	2
Zinc finger CCCH-type with G patch domain-containing protein	JF284508	*Harpegnathos saltator*	1.00E-09	1
Zinc finger protein related gene	JF284563	*Xenopus* (*Silurana*) *tropicalis*	3.00E-51	4
4. Stress				8
Hsp70	JF284547	*Procambarus clarkii*	2.00E-23	2
Hsp70 subfamily B suppressor 1 -like protein-like	JF284600	*Saccoglossus kowalevskii*	6.00E-93	1
Syntenin	JF284543	*Penaeus monodon*	2.00E-14	1
TBC1 domain family, member 4	JF284510	*Homo sapiens*	9.00E-21	1
Tetracycline resistance	JF284480	*Culex quinquefasciatus*	5.00E-14	1
Thioredoxin domain-containing protein 14-like protein	JF284503	*Harpegnathos saltator*	3.00E-35	1
Thioredoxin glutathione reductase	JF284486	*Echinococcus granulosus*	6.00E-40	1
5. Protein synthesis and processing				16
16S ribosomal RNAgene	JF284611	*Cherax quadricarinatus*	7e-120	1
Alkb, alkylation repair homolog 2	JF284562	*Tribolium castaneum*	2.00E-23	1
Calreticulin precursor	JF284491	*Fenneropenaeus chinensis*	6.00E-12 6	1
La protein-like protein	JF284506	*Harpegnathos saltator*	2.00E-19	1
Prefoldin subunit	JF284525	*Nasonia vitripennis*	4.00E-12	1
Proteasome subunit beta type 8	JF284478	*Culex quinquefasciatus*	9.00E-35	1
Protein kinase C-binding protein 1	JF284462	*Camponotus floridanus*	3.00E-67	1
Reticulon-1	JF284464	*Camponotus floridanus*	3.00E-44	1
Ribosome biogenesis protein related gene	JF284552	*Salmo salar*	6.00E-92	2
Ribosomal protein	JF284558	*Scylla paramamosain*	7.00E-53	1
Ubiquitin related protein	JF284553	*Salmo salar*	5.00E-48	5
6. Metabolism and homeostasis				42
2-Phospho-D-glycerate hydrolase	JF284460	*Callinectes sapidus*	1.00E-11	1
Arginine kinase	JF284463	*Camponotus floridanus*	2.00E-47	1
ATP/ADP translocase	JF284532	*Pacifastacus leniusculus*	3.00E-57	1
Bifunctional aminoacyl-trna synthetase	JF284475	*Culex quinquefasciatus*	1.00E-96	1
Chloride intracellular channel isoform 1	JF284458	*Bombyx mori*	7.00E-16	1
Cathepsin D	JF284544	*Penaeus monodon*	2.00E-12	1
Cytochrome c	JF284521	*Marsupenaeus japonicus*	3.00E-36	1
Cytochrome c oxidase subunit	JF284470	*Cherax destructor*	3.00E-10	3
Cytochrome oxidase subunit I	JF284472	*Cherax quadricarinatus*	8.00E-63	1
Cytochrome P450	JF284518	*Carcinus maenas*	1.00E-50	1
Delta-aminolevulinic acid dehydratase	JF284529	*Osmerus mordax*	2.00E-10	1
Ectonucleotide pyrophosphatase/phosphodiesterase family member 1	JF284565	*Xenopus* (*Silurana*) *tropicalis*	1.00E-10	1
Fucose operon protein fucu	JF284536	*Paenibacillus sp. oral taxon 786str.D14*	7.00E-09	1
Glutaminase kidney isoform, mitochondrial	JF284505	*Harpegnathos saltator*	2.00E-21	1
Glutathione peroxidase 6	JF284487	*Equus caballus*	2.00E-25	1
Glutathione S-transferase, putative	JF284517	*Legionella drancourtii LLAP12*	7.00E-22	1
Interstitial collagenase	JF284494	*Galleria mellonella*	4.00E-37	1
L-Asparaginase	JF284479	*Culex quinquefasciatus*	2.00E-15	1
Mg^{2+} and Co^{2+} transporter, putative	JF284516	*Ixodes scapularis*	2.00E-22	1
Mitochondrial malate dehydrogenase 2, NAD	JF284528	*Oryctolagus cuniculus*	4.00E-54	1
Multiprotein bridging factor 1	JF284456	*Bombyx mori*	7.00E-46	1
NADH dehydrogenase subunit	JF284471	*Cherax destructor*	4.00E-86	2
Phosphatase 2C beta	JF284556	*Scylla paramamosain*	9.00E-11i	1
Phosphoenolpyruvate carboxykinase	JF284526	*Nephrops norvegicus*	1.00E-72	1
Phosphoglycerate mutase 1	JF284461	*Camponotus floridanus*	1.00E-98	1
Prolactin regulatory element-binding protein	JF284481	*Culex quinquefasciatus*	2.00E-06	1
Proteasome p44.5 subunit CG10149-PB	JF284561	*Tribolium castaneum*	6.00E-15	1
Saccharopine dehydrogenase	JF284454	*Aedes aegypti*	3.00E-40	1
Selenoprotein	JF284495	*Glossina morsitans morsitans*	3.00E-45	2
Small calcium-binding mitochondrial carrier, putative	JF284601	*Aedes aegypti*	6.00E-81	1
Sodium/potassium-dependent atpase beta-2 subunit	JF284477	*Culex quinquefasciatus*	3.00E-36	1
Sphingomyelin phosphodiesterase, putative	JF284539	*Pediculus humanus corporis*	7.00E-11	2
Steroid reductase	JF284497	*Glossina morsitans morsitans*	2.00E-18	1
Sterol o-acyltransferase	JF284523	*Nasonia vitripennis*	4.00E-24	1
Triosephosphate isomerase	JF284488	*Eriocheir sinensis*	9.00E-12	2
Xaa-pro aminopeptidase	JF284455	*Aedes aegypti*	3.00E-32	1
7. Unknown genes				151

Sequences analysis of 300 differentially expressed, dot-blot screening-confirmed cDNA clones from forward subtracted cDNA library. The SSH clone sequences were searched through NBCI BLAST program, and only E-value lower e-5 were displayed as known protein.

Table 2
Differentially expressed genes from L12 library after WSSV genes.

Genes	Accession no. of L12 genes	Related species	E-value	Clones
1. Immune relevant gene				5
Coatomer gamma subunit, putative	JF284586	*Ixodes scapularis*	6.00E-18	1
Ferritin protein	JF284594	*Scylla paramamosain*	1.00E-29	1
Gabarap protein	JF284569	*Branchiostoma belcheri tsingtauense*	2.00E-39	1
Origin recognition complex subunit 5	JF284579	*Drosophila melanogaster*	3.00E-10	1
Thymosin-repeated protein 1	JF284580	*Eriocheir sinensis*	1.00E-48	1
2. Cytoskeletal system				2
Suppressor of profilin 2	JF284574	*Culex quinquefasciatus*	6.00E-33	1
β-tubulin	JF284571	*Cancer borealis*	5.00E-20	1
3. Signal transduction and gene expression				12
52K active chromatin boundary protein	JF284572	*Culex quinquefasciatus*	1.00E-38	1
COX4 neighbor protein, putative	JF284585	*Ixodes scapularis*	4.00E-34	1
Cell growth-regulating nucleolar protein	JF284589	*Mus musculus*	5.00E-33	1
Eftud2 protein	JF284575	*Culex quinquefasciatus*	2.00E-12	1
DNA polymerase delta subunit 3	JF284577	*Danio rerio*	2.00E-17	1
Elongation factor 2	JF284584	*Homarus americanus*	3.00E-82	1
HistoneH3.3	JF284590	*Oncorhynchus mykiss*	1.00E-66	1
MAP kinse-ERK kinase	JF284567	*Bombyx mori*	5.00E-36	1
HistoneH3.3-like	JF284570	*Callithrix jacchus*	3.00E-25	1
Similar to replication factor C/activator 1 subunit, partial	JF284591	*Ornithorhynchus anatinus*	4.00E-21	1
Similar to RING finger protein 181	JF284596	*Tribolium castaneum*	5.00E-32	1
Translocon associated complex TRAP delta-subunit	JF284566	*Argas monolakensis*	3.00E-10	1
4. Stress				5
Ecdysone-inducible gene L3	JF284578	*Drosophila melanogaster*	3.00E-50	1
Grpe protein	JF284592	*Pediculus humanus corporis*	1.00E-32	1
Matrix metalloproteinase	JF284576	*Culex quinquefasciatus*	2.00E-12	1
Pmp22 peroxisomal membrane protein	JF284573	*Culex quinquefasciatus*	1.00E-38	1
Thioredoxin-like	JF284582	*Gallus gallus*	5.00E-62	1
5. Protein synthesis and processing				6
16S ribosomal RNA gene, partial sequence; mitochondrial	JF284610	*Cherax quadricarinatus*	7e-120	1
Ribosomal protein	JF284587	*Lycosa singoriensis*	5.00E-64	1
Tripartite motif-containing 32	JF284597	*Xenopus (Silurana) tropicalis*	1.00E-07	1
Ubiquitin related protein	JF284593	*Scylla paramamosain*	2.00E-33	3
6. Metabolism and homeostasis				11
Aspartyl-tRNA synthetase	JF284583	*Harpegnathos saltator*	7.00E-48	2
Cytochrome c oxidase subunit	JF284608	*Cherax destructor*	3.00E-10	3
Cytochrome oxidase subunit I	JF284609	*Cherax quadricarinatus*	8.00E-63	1
Cytosolic juvenile hormone binding protein 36 kDa subunit	JF284568	*Bombyx mori*	9.00E-08	1
Dyslexia susceptibility 1 candidate 1	JF284595	*Taeniopygia guttata*	2.00E-16	1
Glucose-regulated protein 78	JF284581	*Fenneropenaeus chinensis*	1.00E-27	1
Phosphoglycerate mutase 1	JF284607	*Camponotus floridanus*	2.00E-97	1
tRNA pseudouridine synthase PuslO-like isoform	JF284588	*Macaca mulatta*	2.00E-07	1
7. Unknown genes				25

Sequences analysis of 66 differentially expressed, dot-blot screening-confirmed cDNA clones from forward subtracted cDNA library. The SSH clone sequences were searched through NBCI BLAST program, and only E-value lower e-5 were displayed as known protein.

L12-254 (an unknown gene) were observed to be highly enhanced after WSSV challenge (Fig. 2). The other genes such as aspartyl-tRNA synthetase (ASPT) and MAP kinase-ERK kinase (MAPK) from L12 showed less up-regulation compared with the genes mentioned above (data not shown). These results together clearly confirmed the SSH efficiency and implied that the transcripts of our isolated genes could be enhanced towards WSSV infection in the red claw crayfish Hpt cell cultures.

4. Discussion

Host defense against WSSV infection is initiated by the innate immune response, operating on the basis of general viral features. Pathogens are recognized through PRRs that detect pathogen associated molecule patterns (PAMPs) and signal the presence of infection to the host, thereby triggering host defense, including antiviral and proinflammatory cytokine generation. The mechanism focused on the WSSV invasion is limited due to a lack of crustacean cell line for WSSV propagation, and the process by which WSSV penetrates cells is not clearly understood. Fortunately, the crayfish Hpt cells are suitable for WSSV replication and could be cultured for several months. Thus, this cell culture is useful for WSSV invasion studies. In the current study, differentially expressed genes related to WSSV infection were successfully isolated, suggesting their possible important roles involved in WSSV pathogenesis. Among these genes, some have been documented to be involved in the anti-WSSV responses such as ALF (Liu et al., 2006), Kazal-type proteinase inhibitors (Cerenius et al., 2010), Ras-related GTPase and ferritin (Pan et al., 2005). While a number of genes like LR (Elrod-Erickson et al., 2000), ALG-2, gabarap protein gene and TRIM32 gene were for the first time to be found in relation to WSSV pathogenesis in crustaceans. Furthermore, with the expression of viral genes, simultaneously, the transcript levels of DHB, MBF1, ALG-2 and L1-635 were clearly up-regulated in the L1 library. This was followed by the expression of FIKK, TARRB and other tested genes (data not shown). In L12 library, the gene expression levels of Coa-γ, gabarap, TRIM32 and L12-254 were highly increased during WSSV infection, which was followed by ASPT, MAPK and other tested genes (data not shown). Besides, the genes reported in the earlier studies, such as ALF, kinase, proteinase inhibitors and antioxidant enzymes, were also found in the present study. To some extent, our data conformed the important role of

these molecules functioning in antiviral responsese in crustaceans (Lan et al., 2006; Liu et al., 2006; Wang et al., 2006; Wang et al., 2007; Zhao et al., 2007). Importantly, more genes related to the viral entry, cytoskeletal rearrangement, cellular transport, and intrinsic signal transduction were newly isolated in the present study. This new information will facilitate the mechanism study on WSSV invading into host cells.

The first group of interesting genes is immune-relevant molecules including ALF (Liu et al., 2006; Tharntada et al., 2009), ALG-2, LR (Elrod-Erickson et al., 2000) and other interesting genes. ALF is a well-known cationic protein originally isolated from the horseshoe crab *Limulus polyphemus* (Aketagawa et al., 1986). In addition to its broad antimicrobial properties against bacteria and fungi, ALF was recently found to interfere with WSSV replication in both crayfish and shrimp (Liu et al., 2006; Tharntada et al., 2008). Silencing of ALF specifically results in higher rates of WSSV replication both in the animals and crayfish Hpt cell cultures. Besides, ALF*Pm*6 was lately reported to respond to yellow head virus infection in the black tiger shrimp (Prapavorarat et al., 2010). In the present study, the up-regulation of crayfish ALF*Cq* suggests its certain role in anti-WSSV infection, which is in agreement with a previous report on the crayfish *P. leniusculus* (Liu et al., 2006). Apoptosis is regarded as a host defense for removing the invading virus and this response has been observed in viral target tissues/organs of WSSV-infected shrimp. Silencing of caspase, the central effector of apoptosis, leads to an increase of WSSV copies indicating that apoptosis plays a crucial role in antiviral processes of shrimp (Wang et al., 2008). A gene involved in apoptotic response, namely ALG-2, was also isolated from our L1 library implying a key role of this molecule on WSSV infection. ALG-2 was originally identified as a proapoptotic calcium binding protein. Nevertheless, a recent study showed that ALG-2 has an anti-apoptotic function in HeLa cells by facilitating the passage through checkpoints in the G2/M cell cycle phase (Hoj et al., 2009). The best characterized target of ALG-2 is AIP1/Alix, which is an adaptor protein proved to bind to many growth-related signaling molecules (Odorizzi, 2006). Recently, it was shown that AIP1/Alix participates in endocytosis related processes like virus budding (Strack et al., 2003) and multivesicular endosome formation. Furthermore, overexpression of either ALG-2 or ALIX could activate the JNK signaling pathway that has essential role in many biological processes, including cell growth, differentiation, apoptosis and immune responses (Tsuda et al., 2006). However, the mechanism of apoptosis induced by infection of WSSV remains largely unknown. Whether ALG-2 and AIP1/Alix also function together in WSSV pathogenesis in crustaceans still needs further investigations. Usually, the process of viral entry relies on the presence of certain specific receptors localized on the surface of the host cell. To date, the cell surface receptor for WSSV is unclear. Given the ability of LR to function as an attachment receptor for a series of viruses including Sindbis virus, Dengue virus and Adeno associate virus (Omar et al., 2011), the possible role of LR as a binding partner for WSSV cannot be excluded. It remains to be elucidated if a direct activation of LR gene by WSSV could contribute to the antiviral phenotype. Further functional study like RNAi of LR in crayfish is on the way.

Host-cell provides a varity of internalization process for virus entry such as receptor mediated endocytosis, including the well described clathrin- and caveolar-dependent pathways. The entry of enveloped viruses differs from non-enveloped viruses in that enveloped viruses typically reach the endosomal compartment by trafficking in clathrin-coated vesicles. Examples of enveloped viruses with entry mechanisms have been well-defined for HCV, influenza A and HIV (Thorley et al., 2010). Interestingly, the transcripts of certain genes related to endosome trafficking machinery and cytoskeletal dynamics, including clathrin, dynein related genes and actins/tublins, were also found to be enhanced upon WSSV challenge in crayfish Hpt cells. Besides, the molecules participated in the cellular proteostasis of actins and tublins were also found to be up-regulated. These molecules include prefodin (PFD), TRIM32, poly-ubquitins and E3 ligases, indicating that WSSV infection leads to the activation of cytoskeletal genes in crayfish Hpt cells. Cytoskeletal proteins like actins and tublins support diverse cellular processes (Lundin et al., 2010). For example, actin and tublin which are able to form microfilaments and microtubles, along with their associated molecular motors like myosin, kinesin and dynein, work together for phagocytosis. As shown in human macrophages, the rearrangement of cytoskeletal proteins was found against influenza A virus infection (Ohman et al., 2009). Importantly, phagocytosis mediated by *Pj*-Rab has been described to play a key role in anti-WSSV response (Wu et al., 2008), and shrimp actin was clearly shown to interact with VP26 of WSSV (Xie and Yang, 2005), suggesting their significance in anti-WSSV pathogenesis. PFD is a jellyfish-shaped molecular chaperone, which is needed for stabilization of nascent cytoskeletal proteins, including the biogenesis of actin and tubulin. PFD acts in substrate delivery as well as promotes the actin and tubulin folding by working as a transient "cap" for CCT that recruits partially folded substrate molecules to the chaperonin for another cycle of assisted folding (Siegers et al., 1999). In the present study, a strong up-regulation of TRIM32 gene was also observed by semi-quantitative RT-PCR in L12 library. TRIM32 is a cytoplasmic ubquitin ligase crucial for RIG-I mediated signaling events by facilitating its interaction with MAVS (Gack et al., 2007). TRIM32 belongs to the RING-family E3 ubiquitin ligase, which is involved in actin degradation by binding myosin in skeletal muscle and ubiquitylates α-actin in vitro (Kudryashova et al., 2005). In human embryonic kidney cells, ectopic expression of TRIM32 results in a reduced level of cytoplasmic β-actin (Kudryashova et al., 2005). Mutations of TRIM32 have been shown in two human diseases including the muscular dystrophy and the ciliary disorder Bardet-Biedl syndrome. However, it is not clear yet for the precise role of TRIM32 in the molecular etiology of these diseases (Chiang et al., 2006; Saccone et al., 2008). The role of TRIM32 in anti-WSSV response is yet unknown which calls for further studies. Taken together, our results suggest that cytoskeleton network might interact with WSSV to regulate antiviral signaling during WSSV infection. Our understanding of the significance of cytoskeletal quality control pathway employed in WSSV invasion is unclear yet. Given the specialized requirement for cytoskeleton rearrangement in the endocytosis, elucidating the details of these molecules will continue to shed light on the importance of the cytoskeleton in WSSV invasion. In addition, further studies involving single-particle tracking of fluorescent-labeled WSSV particle in real-time will help reveal the process of entry of WSSV and offer a greater understanding of the endocytic mechanism active within the host cell, which might be useful for anti-WSSV prevention.

All biological effects within a cell are activated and controlled via cell signaling cues. Because viruses are obligate intracellular parasites, they depend on cell signaling to regulate all events within the cell that control virus replication. For instance, many viruses employ host cell enzymatic machinery such as protein kinase for viral replication. Meanwhile, viruses need host-cell processes for their life cycles and have evolved mechanisms for regulating cellular conditions to an environment conducive to replication while escaping recognition and destruction by the host immune system. Because the viral infection regulates many aspects of cellular physiology, their invasion and replication could result in activation and manipulation of host-cell signaling cascades. It might be expected, therefore, that host signaling pathways under homestasis are affected upon infection, by either viral or host factors. Indeed, we found that some signal transduction genes related to pathogen recognition were obviously up-regulated towards WSSV

infection from our SSH libraries. Interestingly, a large number of genes involved in signal transduction were firstly reported from this study. These genes include checkpoint-like protein, barrier-to-autointegration factor (BAF), kelch domain containing 2-like, WD-repeat protein and MAPK et al. Usually, the virus takes advantage of cellular proteins and pathways to achieve the different steps in its life cycle. For example, the reverse transcribed cDNA of HIV-1 is inserted in the host cell genome for achieving productive infection mediated by the viral integrase. Meanwhile, studies show that the viral integration is assisted by cellular proteins, or co-factors, to gain integration into the infected cell. Nevertheless, BAF, a necessary molecule for DNA binding that is found in both the nucleus and cytoplasm, is capable of blocking autointegration (Van Maele et al., 2006). Given the fact that silencing of BAF by RNAi in *Caenorhabditis elegans* embryos (Zheng et al., 2000) or generation of BAF-null *Drosophila* embryos (Furukawa, 1999) led to lethal phenotypes, BAF is supposed to have a key role in nuclear assembly. Besides, BAF also functions as a potent inhibitor of poxvirus replication unless its DNA-binding activity is suppressed by phosphorylation mediated by the virally encoded B1 protein kinase, implying that BAF prevents replication of exogenous viral DNA in the cytoplasm (Wiebe and Traktman, 2007). However, the role of BAF during WSSV replication remains to be elucidated, considering its up-regulation post WSSV infection in red claw crayfish Hpt cells. In this light, the possibly validated cellular co-factors for viral integration could constitute a promising new target for antiviral treatment. On the other hand, the activation of p38 MAPK by PRRs has been investigated in the context of the antiviral immune responses (Kopp and Medzhitov, 2003). The ERK and MAPKs have been reported to contribute to virus replication in vitro (Jafri et al., 2007; Marchant et al., 2009a,b). Further investigation showed that inhibition of p38 MAPK activation is an effective and novel antiviral strategy in vivo, and the activation of this kinase by some pharmaceutical agents promotes virus replication (Marchant et al., 2009a,b). A recent study indicates that respiratory viruses could usurp host-cell immune responses for the benefit of virus replication through activation of p38 MAPK mediated by a PRR (TLR4) and MyD88 (Marchant et al., 2010). Similarly, WSSV is able to enhance expression of its IE1 gene by annexing a shrimp STAT in host cells (Liu et al., 2007). Besides, two *Litopenaeus vannamei* HMGB proteins were found to interact with transcription factors *Lv*STAT and *Lv*Dorsal to activate the promoter of IE1 gene in a STAT/NF-κB binding sites-dependent manner in *Drosophila* S2 cells (Chen et al., 2011). These data together might imply that viruses have evolved the capability to initiate their entry and replication in parallel with these common responses. This strategy has been successful such that p38 MAPK activation has evolved as a crucial tropism determination of virus replication, rescued in MyD88 KO cells by ectopic activation of p38 (Marchant et al., 2010). For instance, inhibition of p38 MAPK signaling is able to significantly suppress replication of respiratory viruses including Coxsackievirus B3, influenza virus A, human parainfluenza virus type 3, human adenovirus and respiratory syncytial virus. Further investigation on pathways recruited in virus-meidated p38 and extracellular signal-regulated kinase MAPK activation revealed that p38 MAPK is a determinant of virus infection, which is dependent on MyD88 expression and TLR4 ligation (May and Plasterk, 2005). The effects on key kinases related to viral replication by certain inhibitors might be employed as potential therapeutic strategy for antiviral treatment. It is presumed that PRRs such as the Toll-like receptors may recognize high-mannose residues expressed on the surface of viruses, or the virus surface glycoproteins that mediate virus entry into the host cell (Marchant et al., 2010). So far, the specific PRRs or receptors for WSSV entry into host cell are not characterized yet. There is little investigation to date on the functional impact of MAPK pathway in WSSV infection. Whether p38 MAPK is a determinant of WSSV infection and tropism in target host cells still needs further investigations.

Beside the genes discussed above, the other gene groups related to stress, metabolism and homestasis, and unknown genes were also isolated from crayfish Hpt cells post WSSV infection. Further studies on these genes will provide more insights into WSSV pathogenesis. In conclusion, these data together strongly suggest that the viral infection caused a strong host-cell immune responses against WSSV. However, the roles of these molecules isolated by SSH in viral infections remain elusive. Further investigations on these molecules will certainly shed light on the mechanism of WSSV entry as well as searching for efficient anti-WSSV therapy target.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2011.02.015.

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