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Function of shrimp STAT during WSSV infection

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ABSTRACT

JAK/STAT signaling pathway plays key roles in the antiviral immunity of mammals, fish and insect. However, limited knowledge is known about the function of JAK/STAT signaling pathway in the antiviral immunity of shrimp although virus disease has caused severe mortality in shrimp aquaculture. In order to understand the function of JAK/STAT signaling pathway in the antiviral immunity of shrimp, dsRNA interfering technique was used to silence the expression of STAT gene in *Litopenaeus vannamei*, and the mortality of shrimp was detected after WSSV infection. Furthermore, the expressions of some potential target genes regulated by *STAT* or genes related to RNA interfering pathway were detected in *STAT* silenced shrimp during WSSV infection. The WSSV copy number in *STAT* silenced shrimp was 10^2 -10^3 copies/ng DNA which was much lower than that in the control. The mortality in *STAT* silenced shrimp caused by WSSV infection decreased very significantly compared to their controls. The function of STAT was verified *in vitro* cultured cells of hematopoietic tissue of crayfish *Cherax quadricarinatus* by adding specific inhibitor of STAT3(S3I-201), and the cultured cells treated with S3I-201 showed much less WSSV copy number than their controls, which further suggested that STAT might be helpful for the replication of WSSV. Expression analysis on the potential STAT target genes and genes in RNA interfering pathway provide important information for understanding the functional mechanism of STAT in antiviral immunity of shrimp.

1. Introduction

Janus family tyrosine kinase and signal transducer and activator of transcription (JAK/STAT) signaling pathway has been proved to be very important in antiviral process of vertebrate [1,2] and invertebrate [3,4]. In mammals, the activation mechanism of antiviral JAK/STAT pathway is well understood. Infectious virus induces the production of interferon, interleukin *etc*, which is recognized by cytokine receptors, and then leads to the activation of JAK, which in turn phosphorylates the cytoplasmic domain of the receptor to allow recruitment and phosphorylation of STAT. STAT dimerizes and moves to the nucleus to activate the transcription of interferon inducible genes to perform antiviral function [5]. Similarly, the expression of interferon inducible genes can be activated through STAT1 pathway in fish [2,6,7]. In insects, such as mosquitoes and *Drosophila*, JAK/STAT pathway also shows antiviral activity, although no interferon has been demonstrated [3,4,8]. In *Culex* mosquito, activation of JAK/STAT pathway can induce the expression of *Vago*, which is regarded as an antiviral factor, to restrict the West Nile virus infection [4]. In *Drosophila*, the RNA virus infection could activate the transcription of *Vir-1*, *CG12780* and *CG9080* [9,10], and the *jak* mutant flies were susceptible to virus [3]. Contrasted to the extensive study on the role of JAK/STAT to DNA virus in mammals [11,12], studies on its function in insects mainly focused on the antiviral immunity to RNA virus [3,13].

White spot syndrome (WSS) is still the major threat to shrimp aquaculture which usually causes 100% mortality of the shrimp in three to four days [14]. The responsible pathogen for WSS was designated as white spot syndrome virus (WSSV), a double strand DNA virus [15], which has a broad host range including prawn [16] and crayfish [17]. Toll, IMD and JAK/STAT pathways are regarded as three main signaling pathways regulating humoral immunity of shrimp [18]. Most components in the Toll pathway and IMD pathway of shrimp are responsive to WSSV infection, however very limited information is available for the function of JAK/STAT pathway during WSSV infection [18]. STAT, as one key component of JAK/STAT pathway, was isolated and identified in different shrimp species, including *Penaeus monodon* and *Fenneropenaeus chinensis* [19,20]. Shrimp STAT can enhance the expression of WSSV immediate-early gene *ie1 in vivo* [21]. WSSV infection could

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modulate the transcription of *STAT* and increase the protein level of phosphorylated (activated) STAT which translocated from the cytoplasm to the nucleus [19] and further supported the finding that WSSV benefited from STAT activation in shrimp. In order to further learn the function of STAT during WSSV infection, RNAi approach and STAT inhibitor were used to study the function of STAT in crustacean. These data will be very helpful for us to understand the roles of JAK/STAT signaling pathway in antiviral immunity of crustacean.

2. Materials and methods

2.1. Shrimp

Healthy Pacific white shrimp (*Litopenaeus vannamei*) reared in our laboratory with body length of 6.8 ± 0.5 cm and body weight of 4.4 ± 1.0 g were used in this study. Shrimp were acclimated in the aerated seawater at the temperature of 25 ± 1 °C and fed with shrimp food pellet three times a day.

2.2. Reagent and virus

S3I-201, as STAT3 inhibitor, was purchased from Sigma Company and dissolved in dimethyl sulfoxide (DMSO, TaKaRa, China) to a final concentration of 50 μM. Trypan blue was purchased from Sigma Company and dissolved in PBS to the final concentration of 0.4%. WSSV used in the study was purified from the tissues of infected *Exopalamon carincauda* and quantified according to the method previously described [22].

2.3. Primary cultured Hpt cells of crayfish

The hematopoietic tissue (Hpt) cells of crayfish, *Cherax quadricarinatus*, can be infected by WSSV [23]. Healthy freshwater crayfish, free of WSSV, was used to collect Hpt cells as described by Söderhäll et al. [24]. Briefly, a sheet of cell clusters situated on the dorsal side of the stomach was dissected and incubated in 1 mL of 0.1% Type I collagenase (Sigma) and 0.1% Type IV collagenase (Sigma) in CPBS (10 mM Na_2HPO4, 10 mM KH_2PO_4, 0.15 M NaCl, 10 μM $CaCl_2$, 10 μM $MnCl_2$, 2.7 μM KCl; pH 6.8) at room temperature for 45 min, and then centrifuged at 1000 g for 5 min at room temperature. The cell pellets were resuspended and maintained in L15 medium with L-Glutamine (pH 7.2, Sigma) in an incubator with temperature of 26 °C for further use.

2.4. Double strands RNA (dsRNA) synthesis

Forward and reverse primers of *STAT* with T7 promoter sequence (shown in Table 1) were designed according to the reported *STAT* sequence of *L. vannamei* (GenBank: HQ228176) and used to amplify the fragment (414 bp) of *STAT* based on the cDNA template extracted from lymphoid organ (Oka) of *L. vannamei*. The polymerase chain reaction (PCR) was performed as follows: one cycle of 94 °C for 4 min; 35 cycles of 94 °C for 30 s (s), 57 °C for 30 s and 72 °C for 30 s; followed by one cycle of 72 °C for 10 min. The PCR products were purified by QIAquick PCR Purification Kit (QIAGEN, Germany) and used as the template for synthesis of dsRNA of *STAT* with TranscriptAid T7 High Yield Transcription kit (Thermo Fisher Scientific, USA). DsRNA of the *enhanced green fluorescent protein* (*EGFP*, 289 bp) was synthesized based on the template of pEGFP-N1 plasmid using EGFP-F and EGFP-R with the T7 promoter sequences (shown in Table 1). The synthesized dsRNA was treated with ribonuclease (RNase) A (TaKaRa, China) to digest the single strand RNA. Synthesized dsRNAs were monitored by determining the size shift on 1.2% agarose gel during electrophoresis, and the concentration of dsRNAs was measured by NanoDrop 1000 spectrophotometer (Labtech, UK) and stored at −70 °C for later use.

Table 1
Sequence information of primers used in the present study.

Primers	Sequences (5′-3′)	Tm (°C)
STATdsRNA-F	TAATACGACTCACTATAGGGTTGCCAGTTGTAGTCATTGTCC	60
STATdsRNA-R	TAATACGACTCACTATAGGGTTCTTCTGCCTGGCGTCTACC	
EGFP-F	TAATACGACTCACTATAGGGCAGTGCTTCAGCCGCTACCC	55
EGFP-R	TAATACGACTCACTATAGGGAGTTCACCTTGATGCCGTTCTT	
STAT RT-F	TATATCCGAATGTGCCTAAG	56
STAT RT-R	ATAGTTTGTGGTGTGTTGGG	
18S rRNA F	TATACGCTAGTGGAGCTGGAA	55
18S rRNA R	GGGGAGGTAGTGACGAAAAAT	
VP28-F	AAACCTCCGCATTCCTGTGA	56
VP28-R	TCCGCATCTTCTTCCTTCAT	
AGO1 RT-F	CCTCTAACGGATTCCCAGC	55
AGO1 RT-R	ACTTTCTTCGCATCGCACC	
AGO2 RT-F	CAAATCACTACAACAGGAC	57
AGO2 RT-R	GGTCAACAATGGTGCCTGG	
Dicer1 RT-F	CCGGAGATAGAACGGTTCAGTG	56
Dicer1 RT-R	CGATAATTCCTCCCAACACCTG	
Dicer2 RT-F	ATGACACACTTGGGCGAGC	58
Dicer2 RT-R	GCCATGCTACCTTGAGTGA	
GILT RT-F	TCCTTCACCTGCCAACACG	56.5
GILT RT-R	CGAGAGAAGGCAGTTGACG	
TNFSF RT-F	ATCTCCTTCTTGCCACACC	56
TNFSF RT-R	TCCTCCCATCTTCCTTCCC	

2.5. Optimization of the dosage of dsRNA for interference

In order to obtain appropriate silencing efficiency of *STAT* dsRNA, different injection dosages, including 5 μg, 10 μg and 20 μg for each shrimp with body weight of 4.4 ± 1.0 g were set to detect the efficiency of RNA interference. The dsRNA of *EGFP* or *STAT* was separately injected into the last abdominal segment of shrimp and each dose had three replicates. After 48 h of injection, three shrimp in each group were collected separately, and their cephalothoraxes were isolated and frozen in liquid nitrogen for further RNA extraction.

Total RNA were extracted with RNAiso Plus (TaKaRa, China) following the manufacturer's protocol. The extracted RNA was quantified using a Nanodrop ND1000 spectrophotometer. Total RNA was treated with RQ1 RNase–Free DNase (Promega, USA) to remove contaminating DNA. The cDNA was synthesized using Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, USA) following the manufacturer's protocol with Hexamer (NNN NNN) (Sangon, China).

Real-time quantitative PCR (qPCR) was used to test the relative expression level of *STAT*. *18S rRNA* was used as reference gene. The primer sequences used in the present study were shown in Table 1 qPCR detection of *STAT* transcripts for all samples was repeated in triplicate. The expected fragment of *STAT* and *18S rRNA* was of 170 bp and 166 bp in length, respectively. The effectiveness of each pair of primers was analyzed following the method described by Freeman et al. [25]. The qPCR for *18S rRNA* was carried out according to the program as follows: 40 cycles of 95 °C for 15 s, 55 °C for 20 s and 72 °C for 20 s. The qPCR for *STAT* followed almost the same conditions, while the annealing temperature was 56 °C. The relative expression level of the target gene was calculated using the comparative Ct method with the formula $2^{-\triangle\triangle Ct}$ [26]. Unpaired two tails *t*-test and Tukey multiple comparison test were used for statistical analysis by GraphPad Prism software (version 5.0). The *P* value less than 0.05 was considered statistically significant.