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A signal transducers and activators of transcription (STAT) gene from *Scylla paramamosain* is involved in resistance against mud crab reovirus

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ABSTRACT

A STAT gene from *Scylla paramamosain*, named *SpSTAT*, was cloned and characterized. The full length of *SpSTAT* mRNA contains a 5′untranslated region (UTR) of 238 bp, an open reading frame (ORF) of 2388 bp and a 3′ UTR of 326 bp. The *Sp*STAT protein contains four characteristic STAT domains and showed 84% identity (90% similarity) and 44% identity (64% similarity) to *Litopenaeus vannamei* STAT protein and *Human* STAT5a/b protein, respectively. The mRNA of *SpSTAT* was high expressed in the intestine and eyestalk and low expressed in the heart and muscle. Moreover, expression of *SpSTAT* was significantly responsive to challenge of mud crab reovirus (MCRV), Poly(I:C), LPS and *Staphylococcus aureus*. *Sp*STAT could be activated by Poly(I:C) and LPS to translocate to the nucleus of *Drosophila* Schneider 2 (S2) cells. *Sp*STAT could be phosphorylated by interaction with JAK of *S. paramamosain* (*Sp*JAK) and activated to translocate to the nucleus of S2 cells. Furthermore, Silencing of *SpSTAT in vivo* resulted in higher mortality rate of MCRV infected mud crab and increased the viral load in tissues, suggesting that *SpSTAT* could play an important role in defense against MCRV in mud crab.

1. Introduction

The Janus Kinase (JAK)/signal transducers and activators of transcription (STAT) pathway was firstly identified as a cytokine, induced signaling pathway in mammals and has been widely characterized from human to *Drosophila* [1,2]. The JAK/STAT signaling pathway is involve in multiple biology processes, especially mediates several innate immunity processes including activation of neutrophils and macrophages, regulation of inflammatory responses, and antiviral immunity [1,3].

In the canonical mode of JAK-STAT signaling, the ligands binding to transmembrane receptors and the receptors dimerization trigger the activation of the receptor-associated JAK kinases, which in turn phosphorylate tyrosine residues in the cytoplasmic tail of the receptor. These phospho-tyrosine residues function as docking sites for cytoplasmic STAT proteins, which are then phosphorylated by JAK on a crucial C-terminal tyrosine residue around the 700- amino-acid position. Phosphorylated STAT proteins either homo- or heterodimerize via Src-homology 2 (SH2)-domain–phospho-tyrosine interactions and translocate to the nucleus, where they function as transcriptional activators, and bind to a specific DNA motif in the promoters of the target genes to activate transcription [4–6]. Genes regulated by the mammalian JAK-STAT pathway also include positive and negative regulators that modulate the magnitude and/or duration of signaling [7–9]. Activated STAT proteins drive their own expression, forming a positive-feedback loop or compensating for activation-induced STAT degradation [8]. Similarly to mammalian STATs [8], STAT92E (*Dm*STAT) itself is transcriptionally induced by JAK-STAT signaling in *Drosophila* [10].

The JAK/STAT pathway has been proved to be very important in antiviral process of vertebrate [11,12] and invertebrate [13,14]. In mammal, the JAK-STAT signaling pathways are activated by type I IFNs via a type I IFN receptor, and then the activated STAT moves to the nucleus to activate transcription of interferon-stimulated genes (ISGs) to perform antiviral function [15,16]. Similarly, the expression of interferon inducible genes can be activated through STAT1 pathway in fish [12,17,18]. The JAK/STAT signaling pathway induces antiviral activity in insects, including *Drosophila* and mosquitoes, through analogous interferon molecule [13,14,19]. In Culex mosquito, activation of JAK/STAT pathway can induce the expression of Vago, which was

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recently reported to function as an IFN-like cytokine in antiviral immune responses through the JAK–STAT signaling pathway [20], to restrict the West Nile virus infection [14]. Mutants for JAK are susceptible to *Drosophila* C virus (DCV) and cricket paralysis virus (CrPV) and exhibit either no or a weak phenotype for other viruses, including solenopsis invicta virus(SINV), vesicular stomatitis virus(VSV), *Drosophila* X virus(DXV), and invertebrate iridescent virus 6(IIV-6) [13,21–23]. This finding suggests that the JAK/STAT pathway-dependent inducible response is virus specific in *Drosophila*. In crustacea, a similar JAK-STAT signaling pathway was also characterized in shrimp [24–26]. The expression level of the members of the JAK-STAT signaling pathway, such as JAK, SOCS, and STAT, was induced by WSSV or poly(I:C) [26–28]. WSSV infection could activate JAK/STAT pathway and induce the expression of Vago to restrict WSSV genome replication in shrimp [24–29]. *L. vannamei* JAK could play an important role in defense against WSSV [27], and Shrimp STAT was found to be activated in response to WSSV infection [24].

The mud crab, *Scylla paramamosain* is an economically important marine species cultured in Southern China [30], and the total aquaculture production reached 152 thousand tons in 2017 [31]. Since 2004, the “sleeping disease” caused by mud crab reovirus (MCRV) resulted in 70% mortality of culturing mud crab at the affected farms, and has resulted in large economic losses in china [30,32]. Because of lacking acquired immunity generally, innate immunity is central to defense against invading pathogens in invertebrates, such as crustaceans [33]. The innate immune response is mainly mediated by three immune signaling pathways: JAK/STAT pathway, Toll pathway and IMD pathway [4,7]. In crab, the Toll pathway is proved to regulate the expression of anti-lipopolysaccharide factors genes to counter invading microbes [34]. Five homologues of Toll pathway components have been identified in mud crab, including Toll [35], MyD88 [36], Tube, Pelle [37] and TRAF6 [34]. Peroxinectin [38] and Epigallocatechin-3-gallate [39] could inhibit WSSV replication in mud crab. In our previous study, we demonstrated that a Janus kinase, a main protein of JAK/STAT pathway, could play an important role in defense against MCRV in mud crab [40]. In this study, a STAT gene was identified and cloned from *S. paramamosain*, and its mRNA expression and potential functions were examined in MCRV infection.

2. Materials and methods

2.1. Cloning full length of SpSTAT cDNA

Total RNA was extracted from mud crab gills by using RNeasy Mini Kit (QIAGEN, Germany) and reverse transcribed into cDNA by using a PrimeScript™ RT reagent kit with a gDNA Eraser (Perfect Real Time, TaKaRa, Japan). Based on our previously reported high-throughput *S. paramamosain* transcriptome data [41], an EST sequence annotated as a putative *STAT* were retrieved, and it was used to design specific primers (Table 1) for verifying. Four specific primers, *SpSTAT* 5′RACE-A/B and 3′RACE-A/B (Table 1), were designed to obtain the 3′ and 5′ ends of *SpSTAT* cDNA sequences by rapid amplification of cDNA ends (RACE), and PCR thermocycle program was set as described before [40]. The second round semi-nested PCR were performed with *SpSTAT* 5′RACE-C and 3′RACE-C primer (Table 1) using the corresponding first round PCR product (200-fold dilution) as template with a standard PCR program. The same band of the second PCR products were cloned into pMD-19T vector (TaKaRa, Japan) and 12 positive clones were selected for sequencing.

2.2. Sequence and phylogenetic tree analysis

The protein sequences of STAT homologues from other species in the GenBank database were retrieved by BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The protein structural domains of STAT was analyzed using BLAST program. Sequence alignments were performed using MEGA 5.0 software [42] and then visualized by GeneDoc software. Phylogenetic tree was constructed based on the deduced amino acid sequences of *Sp*STAT and other known STAT proteins using MEGA 5.0 software [42] with the neighbor joining (NJ) method, applying the amino acid substitution type and Poisson model and bootstrapping procedure with a minimum of 1000 bootstraps.

2.3. Plasmid constructions

The full length of *STAT* was cloned into the *Eco*R I/*Xho* I sites of pAc5.1/V5-His A and pAc5.1-GFP vectors for expressing V5-tagged or GFP-tagged protein. The full lengths of *JAK* was cloned into pAc5.1/V5-His A for expressing V5-tagged protein as described before [40]. These expression vectors were inserted with a *Drosophila* Kozak consensus sequence (ATCAAA) before the ATG initiation codon for efficient initiation of translation [43]. Primer sequences were listed in Table 1.

2.4. Confocal laser scanning microscopy

Drosophila Schneider 2 (S2) cells were cultured at 28 °C in *Drosophila* SDM (Serum-Free Medium; Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). S2 cells were transfected with GFP-tagged plasmid of *SpSTAT* using FuGENE Transfection Reagent (Promega), and S2 cells transfected with *SpSTAT*-GFP was incubated with 2 μg/ml Poly (I:C) or 1 μg/ml LPS, and 48 h later were stained with 2 μg/ml Hoechst 33342 (Beyotime, China). Finally visualized with confocal laser scanning microscope (Leica TCS-SP5, Germany).

S2 cells were co-transfected equal quality with *SpJAK*-V5 and GFP-tagged plasmid of *SpSTAT* and co-transfected *SpSTAT*-GFP with pAc5.1/V5-His A (as control). After 48 h, Indirect immunofluorescence was used to analyze the subcellular localization of *Sp*JAK and *Sp*STAT. Immunostaining was performed using an anti-V5 antibody (1:500). The cells were then incubated with Alexa Fluor 555-labeled goat anti-rabbit antibody (1:500), then stained with Hochest 33342 (Beyotime, China; 1:1000) and visualized on a confocal laser scanning microscope (Leica TCS-SP5, Germany).

2.5. Phosphorylation analysis of SpSTAT

S2 cells were co-transfected equal quality with V5-tagged plasmid of *SpSTAT* and *SpJAK*-V5 or pAc5.1/V5-His A (as control), and S2 cells transfected with *SpSTAT*-V5 was incubated with Poly(I:C), LPS or PBS (as control). At 48 h post transfection, cell lysates were separated by SDS-PAGE gel and transferred onto PVDF membranes for western blot. The procedure for Western blot analysis was as follows: The total cell were harvested and supplemented with SDS-PAGE sample buffer (100 mM Tris, 4% SDS, 20% glycerol, 5% β-mercaptoethanol, 0.2% bromophenol blue, pH 6.8) and boiled for 10 min. The protein samples were electrophoresed in 10% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare). The membrane was blocked with 5% skim milk in phosphate buffered saline (PBS) for 1 h and then incubated at room temperature with an anti- *L. vannamei* STAT (*Lv*STAT) polyclonal antibody (1:1000), anti-V5 tag monoclonal antibody (1:5000, Abcam) and anti-β-actin antibodies (1:5000, Abcam). The signals were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA).

2.6. Co-immunoprecipitation (Co-IP) assay

S2 cells were grown in 6 cm^2 dishes and transfected with plasmids. The plasmids were transfected into the S2 cells for Co-IP assay with three types of combination (i.e., pAC5.1-*SpJAK*-V5 and pAC5.1-*SpSTAT*-GFP, pAC5.1-*SpJAK*-V5 and pAC5.1-GFP, or pAC5.1-*SpSTAT*-GFP and pAC5.1-V5). At 36 h post-transfection, the cells were incubated with Poly(I:C) or PBS, and 12 h later, cells were harvested and lysed in cell lysis buffer (50 mM Tris, pH 7.8; 150 mM NaCl and 1% Nonidet P-

Table 1
Summary of primers in this study.

Name	Sequence (5′-3′)
spliced and verified	
*Sp*STAT-1F	CTCGACACCATTATTTTTCCATAGAAGA
*Sp*STAT-1R	CTGTTGCTTCATAAAGTTGGTATGGAG
RACE	
*Sp*STAT-3′RACE-A	AGGCACTTTTCTCCTGCGATTCTCT
*Sp*STAT-3′RACE-B	GGTCAACAGAATCTTACCCCAATACTCC
*Sp*STAT-3′RACE-C	GTCTCCACAATGCCTCCCTACAACG
*Sp*STAT-5′RACE-A	TGGCTTCCACCTCTCGGTTCTGT
*Sp*STAT-5′RACE-B	GTGGAGCACCAAGCATATTTTCGTG
*Sp*STAT-5′RACE-C	CTGAGACACGAGAGAGTGAGCATACTGG
Real-time RT-PCR	
*Sp*STAT-Q-F	CACCAGATCAAGGAGTGTGAGCGACA
*Sp*STAT-Q-R	GGTGACAAGTGAGGACAGCAAGCGA
18SrRNA-F	AGTCGTAACAAGGTTTCCGTAGGTG
18SrRNA-R	GCGACCACCCACTATTTGTATTAGC
Absolute real-time quantitative PCR	
S12-Q-F	ATCGGAGGACAACTCACTACCACG
S12-Q-R	CATCTCCCTCGCCATATCCAATCT
Protein expression	
*Sp*STAT-F	GGAATTCATCAAACTCGACACCATTATTTTTCCATAGAAGACCA
*Sp*STAT-R	CCGCTCGAGCTGTTGCTTCATAAAGTTGGTATGGAGGAA
*Sp*JAK-F	GTACCTACTAGTCCAGTGTGGTGGATCAAAATGCTGACCGTCGCCTTCAATGGAG
*Sp*JAK-R	TTCGAAGGGCCCTCTAGACTCGAGCAGGTCCTCCTCCTGAAGTTCCTTCACTTG
dsRNA templates amplification	
DsRNA-*Sp*STAT-T7F	GGATCCTAATACGACTCACTATAGGCTTGGTGCTCCACACACAACTAAT
dsRNA-*Sp*STAT-R	CCATGTGGGGTTATTGGTATCTT
dsRNA-*Sp*STAT-F	CTTGGTGCTCCACACACAACTAAT
dsRNA-*Sp*STAT-T7R	GGATCCTAATACGACTCACTATAGGCCATGTGGGGTTATTGGTATCTT
dsRNA-eGFP-T7F	GGATCCTAATACGACTCACTATAGGACGGCAAGCTGACCCTGAAG
dsRNA-eGFP-R	GACTGGGTGCTCAGGTAGTGG
dsRNA-eGFP-F	ACGGCAAGCTGACCCTGAAG
dsRNA-eGFP-T7R	GGATCCTAATACGACTCACTATAGGGACTGGGTGCTCAGGTAGTGG

40) containing a protease inhibitor cocktail (Sigma, USA). The co-immunoprecipitation (Co-IP) and reciprocal Co-IP assays were performed using anti-GFP and anti-V5 agarose affinity gels (Sigma, USA), respectively, and western blot was performed with mouse anti-V5 and rabbit anti-GFP antibodies (Sigma, USA) and alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Sigma, USA), respectively. A standardized aliquot of each input cell lysate was also examined as control.

2.7. Expression levels of SpSTAT mRNA and immune-challenged

Healthy mud crabs *Scylla paramamosain* with 80–100 g weight each were obtained from a farm (Nansha, Guangdong Province, China) and maintained in the aquarium facilities. The tissues including hemocyte, gill, hepatopancreases, heart, intestine, muscle, ganglion, stomach, eyestalk and brain were sampled and each tissue was pooled from 3 crabs and stored in RNAlater (Ambion, USA). RNA was extracted and reverse transcribed into cDNA as described above. Expression levels of *SpSTAT* were detected by real-time RT-PCR on a LightCycler480 system (Roche) with primers *SpSTAT*-Q-F/R (Table 1), and calculated using the Livak ($2^{-\triangle\triangle CT}$) method after normalization to *S. paramamosain* 18S Ribosomal RNA (18SrRNA, Accession No. KC902763.1).

For challenge experiments, mud crabs were divided into six experimental groups, in which mud crabs were injected swimming leg of mud crab with MCRV at 10^4 copies/g body weight, Lipopolysaccharide (LPS) (purified from *Escherichia coli* 0111:B4, Sigma) at 0.4 μg/g body weight, 0.4 μg/g Polyinosinic:polycytidylic acid (Poly I:C, Sigma), 2×10^4 CFU/g (colony-forming unit) of *Staphylococcus aureus*, and 2×10^4 CFU/g CFU of *Vibrio parahaemolyticus*. In the control group each mud crab was received an injection of 50 μL PBS. Three mud crabs were randomly selected from each group and gills of selected mud crab were sampled at 0, 4, 8, 12, 24, 36, 48, 72 and 96 h post injection (hpi). Total RNA was extracted from mud crabs gills and subsequently reverse transcribed to cDNA, and the *SpSTAT* mRNA was detected by real-time RT-PCR.

2.8. Knockdown of SpSTAT expression

The double stranded RNAs (dsRNAs) of *SpSTAT* and GFP (as a control) were produced *in vitro* transcription using RiboMAX™ Large Scale RNA production System-T7 (Promega, USA) with specific primers (Table 1). The lengths of *SpSTAT* and GFP dsRNA are 551 bp and 499 bp, respectively. The final concentration of dsRNA was diluted with DEPC-water to 400 μg/ml. To investigate the RNA interference efficiency, the experimental groups were injected with 200–250 μL *SpJAK* dsRNA (1 μg/g crab), while the control groups were injected with GFP dsRNA and PBS. To measure the RNA interference efficiency, after 48 h post injection, total RNA were extracted from gills and reverse transcribed into cDNA subsequently as the template for RT-PCR. Relative mRNA level of *SpSTAT* was determined with 18S rRNA as the internal control.

2.9. MCRV challenge experiments in SpSTAT-knockdown mud crab

A total of 160 healthy *S. paramamosain* (average 100 g) were divided into four groups, and received an injection of 250 μL dsRNA (*SpSTAT* dsRNA or GFP dsRNA) solution or PBS. At 48 h later, the surviving mud crabs in the PBS-injected group were mock-challenged with PBS as control and others groups were injected with MCRV (2×10^4 copies/g). Mud crabs were kept in culture flasks for about 5 days following infection. Cumulative mortality was recorded every 8 h. Differences in mortality levels between treatments were tested for statistical significance using the Kaplan-Meier plot (log-rank χ^2 test) using the GraphPad Prism software.

For the MCRV challenge test, a parallel experiment was performed to monitor the MCRV replication. Briefly, gill tissues (0.03 g) were

A

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1    gaggcttggctgggagatacggcggctaaagtgttcgtggccgcggctctgggcgggtgagtgaggcgagagtaatagcagcaccagtac
91   accagtagacatgttgacaccctcccggcgagaagcgtcgtaccggggctctctttaagccccaataaccgtagtgtggacagcactata
1                                                                    M  S  L  W  N  R  A  Q  Q  L  P
181  ttgatgctcagttgggaccatcttactcgacaccattatttttccatagaagaccaaaATGTCCCTTTGGAATAGAGCACAACAGTTGCC
12   P  D  A  L  R  E  V  Q  N  V  Y  G  E  Q  F  P  I  E  V  R  H  Y  L  A  G  W  I  E  D  K
271  TCCAGATGCCCTCCGTGAGGTACAAAATGTATATGGTGAGCAGTTCCCCATTGAGGTACGGCATTACCTTGCAGGATGGATTGAAGACAA
42   M  H  Q  W  N  E  I  D  P  E  N  V  S  H  S  Q  Y  A  H  S  L  V  S  Q  L  I  Q  E  I  E
361  AATGCACCAATGGAATGAGATAGACCCAGAGAATGTGTCGCACAGCCAGTATGCTCACTCTCTCGTGTCTCAGCTCATTCAGGAGATTGA
72   N  K  A  L  N  Y  G  S  N  E  D  L  F  L  V  R  I  R  L  D  E  A  A  N  M  F  K  T  R  Y
451  GAATAAGGCACTCAATTACGGCAGCAATGAGGACCTCTTCTTGGTTCGAATACGCCTTGATGAAGCTGCTAACATGTTTAAGACTCGTTA
102  L  N  S  N  P  L  V  L  V  G  I  I  Q  N  C  L  K  I  E  L  Q  L  V  Q  R  H  E  N  M  L
541  CCTGAATAGCAATCCACTGGTACTGGTGGGCATCATCCAGAACTGCCTGAAGATTGAGCTGCAGTTAGTTCAGCGACACGAAAATATGCT
132  G  A  P  H  T  T  N  M  V  I  E  P  C  A  E  I  V  N  E  L  D  I  L  H  R  R  T  R  E  T
631  TGGTGCTCCACACACAACTAATATGGTGATAGAGCCATGTGCTGAGATAGTCAATGAGCTGGACATCTTGCATCGCCGCACGCGAGAAAC
162  A  D  I  L  R  Q  L  E  Q  E  Q  E  S  F  A  L  Q  Y  H  D  C  T  K  I  N  A  H  L  S  H
721  AGCCGACATACTCAGGCAGTTGGAGCAGGAGCAGGAATCCTTTGCTCTTCAGTACCATGACTGTACAAAAATTAATGCTCATCTTTCACA
192  I  Q  S  Q  E  K  T  P  Q  N  R  E  V  E  A  N  L  R  R  R  K  Q  L  G  E  Q  Q  L  T  E
811  TATTCAAAGTCAGGAGAAGACTCCACAGAACCGAGAGGTGGAAGCCAACCTGAGGAGAAGAAAGCAGCTGGGTGAACAGCAACTCACAGA
222  K  V  S  G  L  L  Q  R  R  M  D  L  A  E  K  H  K  G  T  I  D  R  L  N  I  L  Q  Q  R  I
901  GAAGGTGTCAGGATTGCTGCAGAGGAGAATGGACTTGGCAGAGAAGCACAAAGGAACCATTGACAGACTAAATATCCTGCAACAGCGTAT
252  L  D  E  E  L  I  N  W  K  R  E  Q  Q  M  A  G  N  G  K  P  F  N  G  N  K  L  D  T  I  Q
991  ACTTGATGAAGAACTGATCAACTGGAAGAGAGAACAGCAGATGGCGGGGAACGGAAAACCTTTCAATGGAAACAAACTTGACACCATACA
282  E  W  C  E  A  L  A  E  I  I  W  L  N  R  H  Q  I  K  E  C  E  R  H  Q  T  K  I  P  I  T
1081 AGAATGGTGTGAAGCCTTGGCAGAAATTATATGGCTGAACCGGCACCAGATCAAGGAGTGTGAGCGACACCAGACCAAGATACCAATAAC
312  P  H  G  G  V  D  M  L  P  T  L  N  S  H  I  T  R  L  L  S  S  L  V  T  S  T  F  I  I  E
1171 CCCACATGGAGGAGTGGACATGCTGCCCACCCTCAACTCACACATCACTCGCTTGCTGTCCTCACTTGTCACCAGCACATTTATCATTGA
342  K  Q  P  P  Q  V  M  K  T  N  T  R  F  S  A  T  V  R  L  L  V  G  G  K  L  N  V  N  M  T
1261 AAAGCAACCTCCTCAAGTCATGAAGACAAACACCAGATTCTCAGCCACTGTCAGACTGTTGGTGGGTGGCAAGCTCAATGTGAACATGAC
372  P  P  Q  V  R  V  S  I  I  S  E  A  Q  A  N  A  L  L  K  N  D  Q  M  S  K  G  E  M  S  G
1351 CCCACCTCAAGTACGAGTGTCTATCATCAGTGAAGCACAGGCCAATGCTCTTCTGAAGAATGACCAGATGAGCAAAGGTGAAATGTCCGG
402  E  I  L  N  N  T  G  T  M  E  Y  H  Q  S  S  R  Q  L  S  V  S  F  R  N  M  Q  L  R  K  I
1441 AGAAATTCTCAATAATACTGGTACCATGGAGTATCACCAGAGCTCAAGGCAACTCTCTGTAAGCTTCCGCAACATGCAGCTTCGAAAAAT
432  K  R  A  E  K  K  G  T  E  S  V  M  D  E  K  F  S  L  L  F  Q  S  Q  F  S  V  G  G  G  E
1531 TAAAAGGGCTGAGAAGAAAGGAACAGAATCAGTAATGGATGAGAAGTTTTCCCTCCTCTTCCAGTCACAGTTCAGTGTTGGTGGTGGTGA
462  L  V  F  Q  V  W  T  L  S  L  P  V  V  V  I  V  H  G  N  Q  E  P  H  A  W  A  T  V  S  W
1621 ACTGGTTTTCCAGGTGTGGACTCTGTCGCTGCCTGTTGTGGTGATTGTTCATGGTAACCAGGAGCCCCACGCCTGGGCCACGGTGTCCTG
492  D  N  A  F  A  E  Q  G  R  I  P  F  T  V  P  D  K  V  P  W  P  Q  V  A  D  M  L  D  A  K
1711 GGACAATGCATTTGCTGAGCAGGGGCGCATACCATTTACAGTACCTGACAAGGTGCCTTGGCCACAAGTTGCTGACATGTTGGATGCTAA
522  F  K  S  A  T  G  R  G  L  T  E  D  N  L  R  F  L  A  G  K  A  F  R  L  D  R  S  P  Q  V
1801 ATTCAAGTCTGCCACAGGACGAGGATTGACAGAAGATAATCTAAGGTTTTTGGCAGGGAAGGCCTTCAGATTAGACAGAAGTCCCCAAGT
552  Q  D  F  T  N  M  M  L  S  W  S  Q  F  C  K  E  P  L  S  E  R  N  F  T  F  W  E  W  F  F
1891 ACAAGATTTCACAAATATGATGTTGTCCTGGTCACAGTTCTGCAAGGAGCCTCTGTCAGAACGCAACTTTACGTTCTGGGAATGGTTCTT
582  A  V  M  K  V  T  K  E  H  L  R  Q  P  W  N  D  G  S  I  M  G  E  V  G  R  R  P  A  E  E
1981 TGCTGTTATGAAGGTGACCAAGGAACACCTGCGCCAGCCCTGGAATGATGGCTCCATCATGGGTTTTGTGGGGCGTCGACCTGCTGAAGA
612  M  L  K  N  S  K  S  G  T  F  L  L  R  F  S  D  S  E  L  G  G  V  T  I  A  W  M  Y  E  D
2071 AATGTTGAAAAATTCCAAGTCAGGCACTTTTCTCCTGCGATTCTCTGACTCAGAACTTGGAGGAGTCACAATTGCATGGATGTATGAAGA
642  T  S  K  A  G  D  Q  R  D  V  F  M  I  Q  P  E  T  S  K  S  F  A  I  R  P  I  A  D  V  I
2161 TACCAGTAAAGCTGGTGATCAGAGGGATGTGTTCATGCTGCAGCCCTTCACCAGTAAATCCTTTGCCATCCGTCCATTGGCTGATGTGAT
672  A  D  L  K  Y  L  L  Y  L  V  P  N  I  P  K  E  Q  V  F  G  K  Y  Y  T  P  I  G  E  H  P
2251 TGCAGACTTGAAGTACCTCCTCTACTTGTACCCAAATATCCCCAAAGAGCAGGTGTTTGGCAAGTACTACACACCCATAGGGGAGCATCC
702  T  N  N  G  Y  V  K  P  H  L  I  T  H  V  P  G  W  P  G  R  G  S  T  E  S  Y  P  N  T  P
2341 CACCAACAATGGTTATGTAAAGCCTCACCTCATTACCCATGTCCCAGGGTGGCCAGGGCGAGGGTCAACAGAATCTTACCCCAATACTCC
732  Q  P  M  Y  P  M  H  D  T  S  L  G  D  P  P  S  V  S  S  N  P  S  D  C  V  S  T  M  P  P
2431 ACAGCCCATGTATCCCATGCATGACACAAGCCTGGGTGACCCTCCTTCTGTCAGCTCCAATCCTTCTGATTGTGTCTCCACAATGCCTCC
762  Y  N  D  T  D  Y  P  D  I  L  E  N  L  P  D  P  D  F  T  N  F  S  L  D  F  L  H  T  N  F
2521 CTACAACGATACTGATTATCCTGACATCCTGGAAAACCTGCCTGACCCAGACTTCACTAACTTCAGCCTCGACTTCCTCCATACCAACTT
792  M  K  Q  Q  *
2611 TATGAAGCAACAGTAAagttagaaccatccctctttgacatgtataatttttctagattttatattgatgttatattatttgggaatgtg
2701 atgggtgttgattttgttgctcataaattgttcaaatggcaacctgtttatagtgaaaattaacattgcttcattcacattgccctcagt
2791 aaaggaaattatttgatagtacatgaatccttttcaaagtagattgctgttaattcttttgggtattttatattgagagaattgttctaa
2881 taactattgtagtggacatcaaactatagagccaagttacgaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
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B

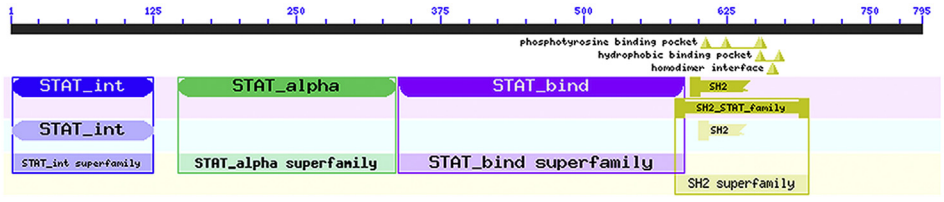

Fig. 1. Sequence analysis of *SpSTAT* gene. (A) Nucleotides and amino acids were numbered on the left of the sequences. The ORF of the nucleotide sequence was shown in upper-case letters, while the 5′ and 3′UTR sequences were shown in lowercase. Amino acid sequence was represented with one-letter codes above the nucleotide sequence. The protein interaction domain in the N-terminal was underlined, the all-alpha domain was boxed with black line, DNA binding domain was shaded; and the Src homology 2 (SH2) domain was marked with green wavy line. (B) Domain structure of *Sp*STAT. *Sp*STAT proteins have four distinct domains, consisting of the protein interaction, all-alpha, DNA binding and SH2 domain. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

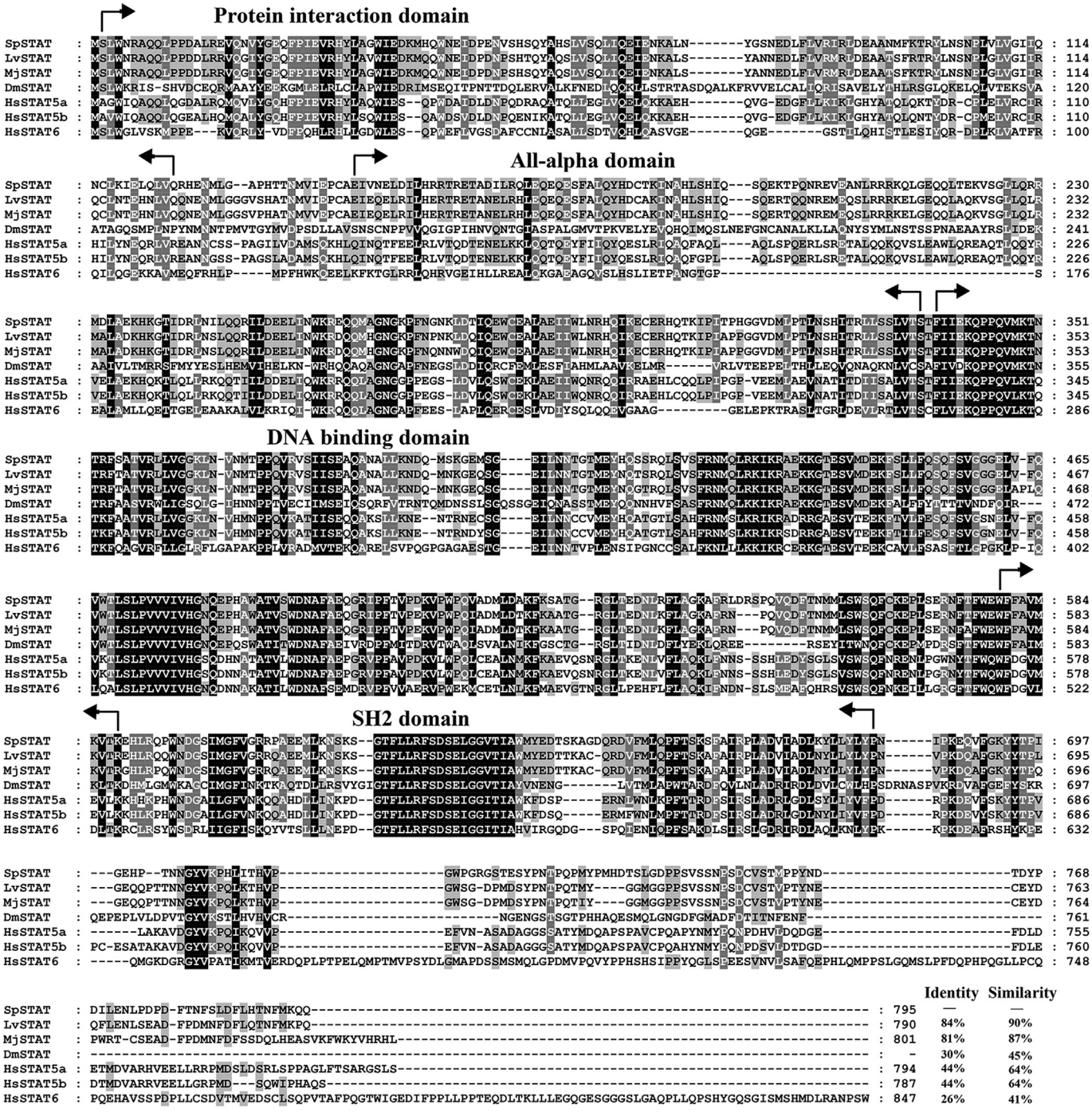

Fig. 2. Multiple sequence alignment of the STATs from human, *drosophila* and crustaceans. The identical amino acid residues shaded in black and the similar residues in gray. Proteins analyzed listed below: LvSTAT, *Litopenaeus vannamei* STAT (ADQ43368.1); DmSTAT92E, *Drosophila melanogaster* STAT (AAC46984.1); MjSTAT, *Marsupenaeus japonicas* JAK(ANA91282.1); HsSTAT5a, *Homo sapiens* STAT5a (NP_003143.2); HsSTAT5b, *H. sapiens* STAT5b (NP_036580.2); HsSTAT6, *H. sapiens* STAT6 (NP_003144.3).

sampled from 9 surviving mud crabs at 48, 72 and 96 h post infection and subjected to total RNA extraction. MCRV copies were measured by absolute real-time quantitative PCR using primers S12-Q-F/R (Table 1) for MCRV S12 gene as previous report [3]. The standard curve was generated from serial dilutions (10^{10}, 10^{9}, 10^{8}, 10^{7}, 10^{6}, 10^{5}, 10^{4}, 10^{3} copies) of plasmid containing S12 fragment. The reaction system and procedure of PCR was performed as follow the real-time RT-PCR. Absolute qPCR was carried out at three replicates per sample. The MCRV copy numbers in 1 g of mud crab gill were then calculated.

3. Results

3.1. Cloning and characterization of SpSTAT

The full length of *SpSTAT* mRNA was 2952 bp long, consisting of a 238 bp 5′- untranslated region (UTR), a 326 bp 3′-untranslated region, and a 2388 bp ORF encoding a 795 amino acids protein with a calculated molecular weight of 91.29 kDa (GenBank Accession MH924352) (Fig. 1A). Conserved domain analysis showed that *Sp*STAT contained a protein interaction domain of 124 amino acids in the N-terminal region,

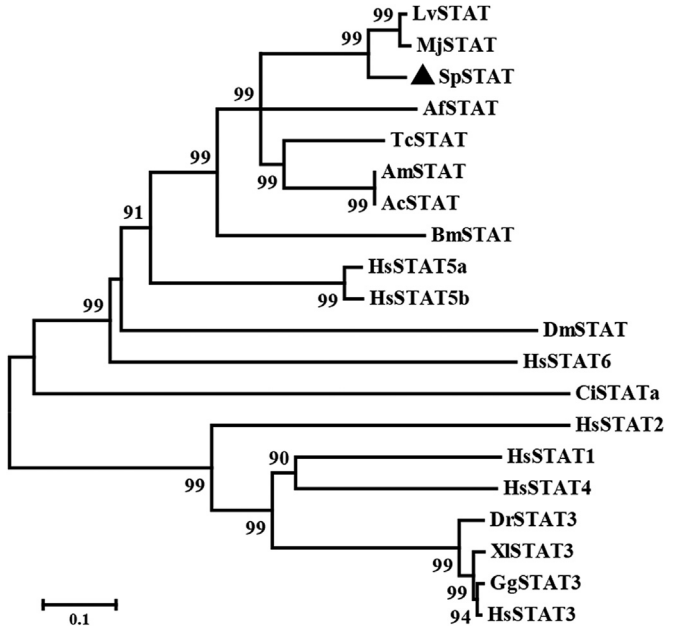

Fig. 3. Phylogenetic tree analysis of the full-length amino acid sequences of STAT proteins from various species using MEGA 5.0 software. SpSTAT was marked with a triangle. Proteins analyzed listed below: SpSTAT, *Scylla paramamosain* STAT (Accession No. MH924352); AcSTAT, *Apis cerana* STAT (PBC29396.1); AmSTAT, *Apis mellifera* STAT (XP_397181.1); TcSTAT, *Tribolium castaneum* STAT (EFA04581.1); LvSTAT, *Litopenaeus vannamei* STAT (ADQ43368.1); MjSTAT, *Marsupenaeus japonicus* STAT (ANA91282.1); AfSTAT, *Artemia franciscana* STAT (ACJ63721.1); BmSTAT, *Bombyx mori* STAT (NP_001157388.1); DmSTAT, *Drosophila melanogaster* STAT (AAC46984.1); CiSTAT, *Ciona intestinalis* STAT (XP_009857877.1); HsSTAT1, *Homo sapiens* STAT1 (AAH02704.1); HsSTAT2, *H. sapiens* STAT2 (AAH51284.1); HsSTAT3, *H. sapiens* STAT3 (P40763.2); HsSTAT4, *H. sapiens* STAT4 (NP_003142.1); HsSTAT5a, *H. sapiens* STAT5a (NP_003143.2); HsSTAT5b, *H. sapiens* STAT5b (NP_036580.2); HsSTAT6, *H. sapiens* STAT6 (NP_003144.3); DrSTAT3, *Danio rerio* STAT3 (AAH68320.1); XlSTAT3, *Xenopus laevis* STAT3 (AAI69720.1); GgSTAT3, *Gallus* STAT3 (AAT64887.1).

all-alpha domain of 191 amino acids located at 146–336, a DNA binding domain of 191 amino acids located at 338–588 and a Src homology 2 domains (SH2) located at 579–696 in the C-terminal region (Fig. 1). The amino acid sequences of *Sp*STAT were aligned with STAT family members as shown in Fig. 2. *Sp*STAT showed 84% identity (90% similarity) to the *Lv*STAT, 30% identity (45% similarity) to the *Drosophila melanogaster* STAT protein and 44% identity (64% similarity) to the *H. sapiens* STAT5 protein (HsSTAT5) (Fig. 2).

3.2. Phylogenetic analysis of the SpSTAT protein

Phylogenetic tree was constructed using the neighbor-joining (NJ) method. The full lengths sequences of STAT family proteins from human, *Drosophila* and other species were phylogenetically analyzed. The constructed phylogenetic tree indicated that *Sp*STAT was nearly clustered with the crustacean proteins *Lv*STAT, *Artemia franciscana* STAT (*Af*STAT) and *Marsupenaeus japonicus* STAT (*Mj*STAT), especially was mostly clustered with the decapoda proteins *Lv*STAT and *Mj*STAT (Fig. 3). The constructed phylogenetic tree indicates that the STAT proteins used in this study were basically separated into the Insecta, Crustacea, Urochorda, and Vertebrata groups, amazedly *H. sapiens* STAT5a and STAT5b are more approached to crustacea than *D. melanogaster* STAT (Fig. 3).

3.3. Subcellular localization analysis

The subcellular localization of *Sp*STAT was observed using confocal laser scanning microscopy. *Sp*STAT was mostly dispersedly presented in the cytoplasm except for a very small amount in the nucleus, suggesting that *Sp*STAT was cytoplasmically localized in normal (Fig. 4A).

To determine whether Poly(I:C), LPS and *Sp*JAK activate *Sp*STAT to translocate from the cytoplasm to the nucleus in S2 cell. S2 cells transfected with *Sp*STAT-GFP was incubated with Poly(I:C) or LPS and co-transfected *Sp*JAK-V5, and then analyzed the subcellular localization of *Sp*STAT. The results indicated that *Sp*STAT were largely presented in the nucleus after dealt with Poly(I:C) and LPS (Fig. 4A), and dispersedly presented in all cell and largely translocate to the nucleus in S2 cell after co-transfected with *Sp*JAK (Fig. 4B), suggesting that *Sp*JAK, Poly (I:C) and LPS could activate *Sp*STAT and translocate from the cytoplasm to the nucleus in S2 cell.

3.4. Effects of SpJAK on SpSTAT phosphorylation

In mammals, STATs are activated by tyrosine phosphorylation prior to execute their biological roles in gene transcriptional activation. To analyze whether *Sp*JAK, Poly(I:C) and LPS could regulate the phosphorylation of *Sp*STAT, S2 cells were co-transfected with V5-tagged plasmid of *Sp*STAT and *Sp*JAK-V5 or incubated with Poly(I:C) and LPS. Due to the lack of the phosphotyrosine antibody specific for *Sp*STAT, phosphotyrosine antibody specific for *Lv*STAT was used to analyzed the phosphorylation status of *Sp*STAT. The results of western blot shown that band of *Sp*STAT phosphorylation was detected after co-transfected *Sp*JAK, but not in the control (Fig. 5A), and the V5-tagged plasmid of *Sp*JAK, *Sp*STAT were all expressed in transfected S2 cells, respectively (Fig. 5B). These suggested that *Sp*JAK could activated *Sp*STAT to be phosphorylated in S2 cells. There are no band of *Sp*STAT phosphorylation after Poly(I:C) and LPS challenged in S2 cells (Fig. 5A).

3.5. Interaction between SpJAK and SpSTAT

The results of subcellular localization and phosphorylation indicate that *Sp*JAK was able to interact with *Sp*STAT. We analyzed the interaction between crab *Sp*JAK and *Sp*STAT by Co-IP. The V5-tagged *Sp*JAK but not the control V5 was co-precipitated by the GFP-tagged *Sp*STAT (Fig. 6). The interaction between *Sp*JAK and *Sp*STAT was further confirmed by reciprocal Co-IP, which showed that slight co-precipitated stain was detected between the V5-tagged *Sp*JAK and GFP-tagged *Sp*STAT but not GFP (Fig. 6). These results suggest that the interaction between *Sp*JAK and *Sp*STAT could be enhanced by Poly(I:C) (Fig. 6).

3.6. Expression of SpSTAT in healthy and immune-challenged mud crab

In healthy mud crab, the expression of *SpSTAT* was high in intestine and eyestalk, moderate in most tested tissues including stomach, brain, hemocyte, gill and nerve, and low in muscle, hepatopancreas and muscle (Fig. 7A). After Poly(I:C) challenge, in mud crab gills, the expression of *SpSTAT* was obviously downregulated at 4 h and increased to the baseline at 8 h and then increased and reached a peak of 1.38-fold at 24 h, then decreased at 36 h and recovered the baseline level during 48–96 h (Fig. 7B). Upon MCRV challenge, *SpSTAT* expression was increased and reached a peak of 1.76-fold at 4 h, decreased the baseline at 8 h and reached lowest level at 12 h, whereafter, increased and reached a peak of 1.72-fold at 36 h, then decreased and maintained a constant expression profile during 72–96 h (Fig. 7C). In response to LPS challenge, the expression level of *SpSTAT* maintained the baseline during 4–12 h and obviously decreased at 24 h. *SpSTAT* expression regain baseline level at 36 h, then decreased to under the normal level at 48 h, whereafter, obviously upregulated at 72 h and slightly downregulated 96 h (Fig. 7D). The *SpSTAT* expression maintained baseline level transitorily, then decreased to lower the baseline during 8–24 h, whereafter, increased and reached a peak at 48 h, decreased to the lowest level at 96 h post *S. aureus* challenge (Fig. 7E). During the *V. parahaemolyticus* challenge, *SpSTAT* expression not change at 4 h, soon

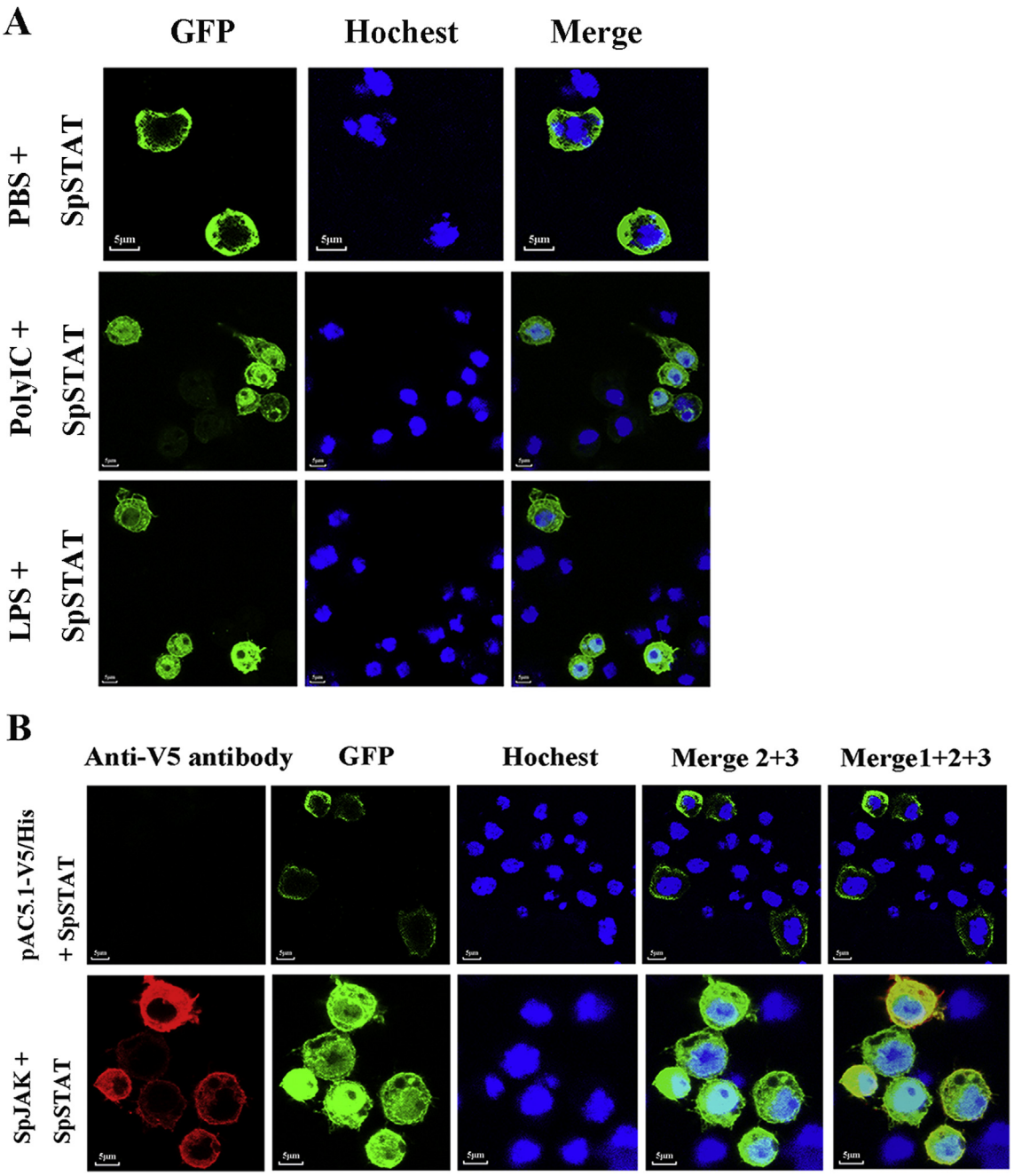

Fig. 4. Subcellular localization of *Sp*STAT. (A) S2 cells transfected GFP-tagged *Sp*STAT were incubated with Poly(I:C), LPS and PBS. At 48 h cells were observed using a Leica laser scanning confocal microscope. The cell nucleus was stained with Hoechst33342 (blue). (B) S2 cells were co-transfected GFP-tagged *Sp*STAT and *Sp*JAK-V5 or the control pAc-V5. At 48 h, immunostaining was performed using an anti-V5 antibody to combine with *Sp*JAK, then incubated with Alexa Fluor 555-labeled antibody, the localization of GFP-tagged *Sp*STAT was observed using a Leica laser scanning confocal microscope. The nucleus was stained with Hoechst 33342 (blue). The merged image represents the digital superimposition of green and blue signals and three signals. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

afterwards obviously upregulated at 8 h, then recovered baseline level at 12 h, and downregulated at 24 h. *SpSTAT* expression maintained baseline level at 24–72 h, and downregulated at 96 h (Fig. 7F).

3.7. Silence of SpSTAT affects mortality of MCRV-infected S. paramamosain and MCRV replication

RNA interference assay was carried out to investigate *SpSTAT* function in *S. paramamosain*. The silencing efficiency of *SpSTAT* was evaluated on mRNA transcript level using qPCR. The results indicated that the mRNA level of *SpSTAT* was down-regulated at 48 h with 56.25% decrease after *SpSTAT* dsRNA injection, and with 31.5% decrease after injection of the GFP dsRNA in the gills (Fig. 8A).

Experimental mud crabs were challenged with MCRV at 2 days post *SpSTAT* dsRNA injection in the following experiments. During MCRV infection, cumulative mortality in the *SpSTAT* dsRNA group was significantly higher than that in the GFP dsRNA group and the gap increased continually from 40 h lasted to 96 h post- MCRV injected, and then the differences reduced at 128 h (logrank c2: 12.43, $P < 0.001$) (Fig. 8B). The mortality rates were 80.8%, 36.7% and 56.7% at 72 h, and final mortality rates were 96.2%, 76.7% and 90.0% for the *SpSTAT* dsRNA, GFP dsRNA and PBS groups, respectively. To further evaluate

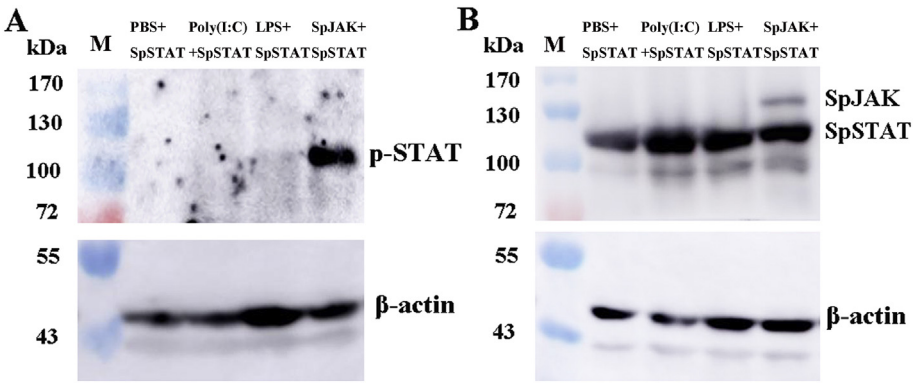

Fig. 5. Effects of Poly(I:C), LPS and *Sp*JAK on *Sp*STAT phosphorylation. S2 cells were co-transfected with V5-tagged *SpSTAT* in combination with *Sp*JAK or pAC5.1-V5/His (as control), and combination with Poly(I:C), LPS. At 48 h post transfection, cell lysates were separated by SDS-PAGE gel and transferred onto PVDF membranes for western blot. A) phosphorylation analysis of *Sp*STAT, incubated with anti-*Lv*STAT polyclonal antibody and anti-β-actin antibodies. B) incubated with anti-V5 tagged antibody and anti-β-actin antibodies. M: 170kDA protein marker.

the effect of *SpSTAT* knockdown on MCRV replication in mud crabs, the virus copies of MCRV in gills were detected using absolute quantitative real-time PCR (Fig. 8C). The virus loads in the *SpSTAT* dsRNA group were significantly higher than those of the GFP dsRNA MCRV control group with 112.33, 36.00 and 28.62-fold at 48 h, 72 h and 96 h, respectively (Fig. 8C). In addition, both the MCRV genome copies and cumulative mortality in the PBS control group were much higher than those of the GFP dsRNA group (Fig. 8B and C). The MCRV genome copies in the *SpSTAT* dsRNA group were obviously higher than those of the PBS control group at 48 h ($P < 0.01$), 96 h ($P < 0.01$) but show no significantly different at 72 h ($P > 0.05$). The mortality rate in the PBS control group is not significantly different with that in the GFP dsRNA group ($P > 0.05$), and is close to the *SpSTAT* dsRNA group. These results suggested that *SpSTAT* could play an important role in antiviral defense against MCRV.

4. Discussion

In this study, the full length of *STAT* was firstly identified and cloned from *S. paramamosain.* Conserved domain analysis showed that *Sp*STAT contained typical domains of STAT, which are a protein interaction domain, all-alpha domain, DNA binding domain and SH2 domain. This is similar with STAT92E [44], *Lv*STAT, *Fc*STAT [45] and *Af*STAT [46]. *Sp*STAT showed very highly homologous to *Lv*STAT and STAT92E. Similarity with STATs of others crustacea [44–46], *Sp*STAT is highly homologous to *Hs*STAT5 in *Homo sapiens* STATs. In the canonical mode of JAK-STAT signaling, STAT molecules are activated to phosphorylate by JAK and dimers translocate to the nucleus [4]. In this study, *Sp*STAT largely translocated to the nucleus after being stimulated by *Sp*JAK, poly(I:C) and LPS (Fig. 4), while were mainly cytoplasmically localized in normal. Phosphorylation and interaction analysis suggests that *Sp*STAT could be activated to phosphorylated by interaction with *Sp*JAK in S2 cells. These are consistent with that STAT92E could be activated to phosphorylated by *Drosophila* HOP and translocate to the nucleus [10]. These indicate that *Sp*STAT, similar with STAT92E, has the possibility to play a role as transcriptional activators. These prompted us to address the possibility that, as other STATs, *Sp*STAT may have the functions of homologous STATs in mud crabs.

In mammals, the JAK/STAT signaling pathway was first identified due to the IFNs-mediated biological responses and found to play critical roles in regulating ISGs [47]. In *Drosophila*, Hopscotch participated in the control of the DCV load in infected flies and was required but not sufficient for inducing virus-regulated genes, such as Turandot M (TotM) and virus-induced RNA 1 (vir-1) [48]. In shrimps, knockdown of *LvJAK*, *LvSTAT*, and effectors *TEP1* and *TEP2* caused an impaired antiviral defense and resulted in high cumulative mortality rates and WSSV copies in tissues [27,49]. Overall, these results suggested that the JAK/STAT pathway plays positive roles in antiviral immunity in invertebrate. It has been reported that there were nucleic acids induced antiviral immunity in invertebrates [50,51]. As in vertebrates, non-specific dsRNA could induce antiviral immunity in the marine shrimp *L. vannamei* [52]. Injection of any dsRNA, whether representing shrimp genes or nonspecific dsRNA controls, resulted in increased survival to TSV and WSSV challenge in shrimps [27,51–53]. In shrimps, GFP dsRNA injected had increased survival to WSSV challenge and reduced WSSV copies [27,51,53]. In this study, similar to shrimps, the MCRV genome copies and cumulative mortality in the PBS control group are higher than those in the dsRNA-GFP group. This prompted us to address the possibility that, as in shrimps, injection of any dsRNA can induce an antiviral immunity in mud crabs. In the present study, the cumulative mortality rates and virus copies of *SpSTAT*-knockdown mud crab are higher than those of the GFP dsRNA (the control group) injected mud

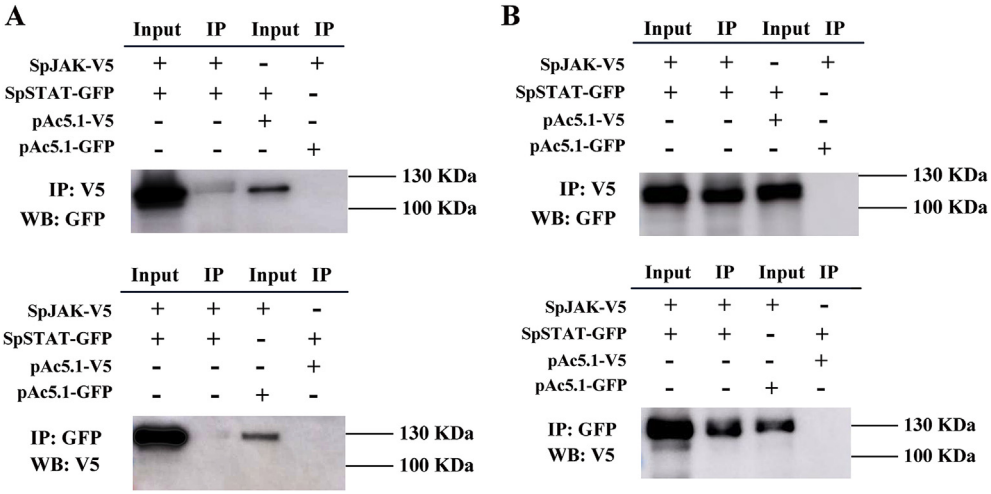

Fig. 6. Interaction between *Sp*JAK and *Sp*STAT. The V5-tagged *Sp*JAK and GFP-tagged *SpSTAT*/GFP (as control) were expressed in S2 cells. At 36 h post-transfection, the cells were incubated with PBS (A) or Poly(I:C) (B). The interaction between *Sp*JAK and *Sp*STAT was analyzed using Co-immunoprecipitation (Co-IP) and reciprocal Co-IP. Input: western-blotting analysis of the input cell lysates before immunoprecipitation.

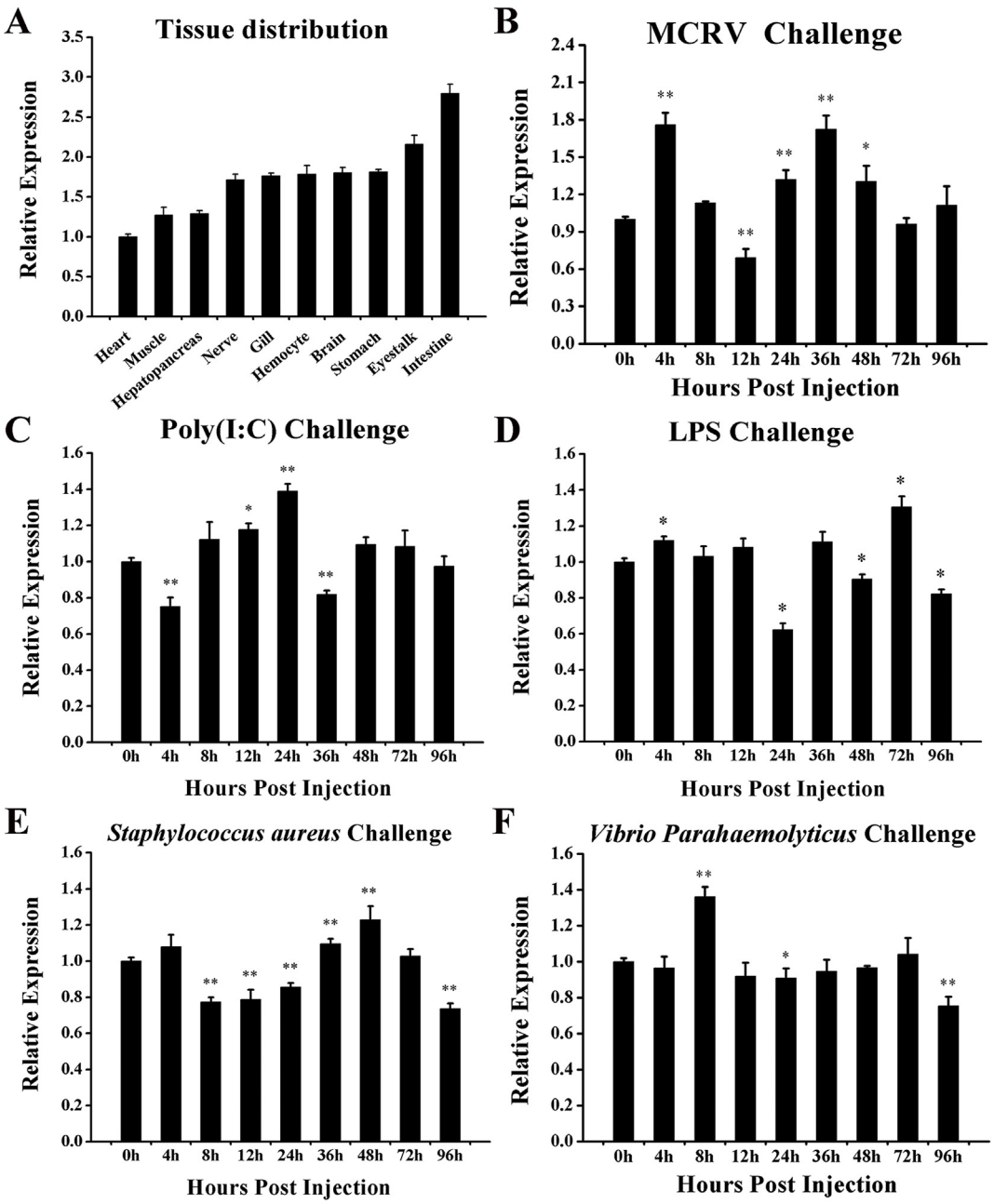

Fig. 7. Tissue distribution of *SpSTAT* in healthy mud crabs and expression profiles of *SpSTAT* after immune challenges. Real-time PCR was performed to detect expression of *Sp*STAT using the Livak ($2^{-\triangle\triangle CT}$) method. Data were normalized to those of 18SrRNA and provided as the means ± SD of triplicate assays. (A) Tissue distribution of *SpSTAT* in healthy *S. paramamosain*. The lowest expression level in the muscle was used as control and set to 1.0. (B–F) Expression profiles of *SpSTAT* in gills from Poly(I:C) (B), MCRV (C), LPS (D), *S. aureus* (E) and *V. parahaemolyticus* (F) challenged mud crabs. Expression level detected at 0 h post injection of each group was set as 1.0. *, $p < 0.05$, **, $p < 0.01$.

crabs after MCRV infection. These results suggest that *SpSTAT* could play an important role in antiviral defense against MCRV. In previous study, dsRNA-mediated gene silencing was suggesting that *SpJAK* plays a major role in antiviral defense against MCRV in mud crab [40]. These results suggest that the complete set of JAK/STAT proteins, similar to *Drosophila* [48] and shrimps [27,49], could activate the JAK/STAT pathway to restrain MCRV in mud crab.

Analysis of *SpSTAT* specific transcriptional regulation and cellular responses was performed following immunostimulant exposure to further understand the defense mechanisms of mud crab. The expression of genes related to the JAK/STAT signal pathway changed after administering various immunostimulants. The expression of the JAK and STAT family members of mandarin fish were increased to certain extent by poly(I:C) in MFF-1 cells [54]. The expression of *LvJAK* was prominently upregulated after the stimulation of poly(I:C) and WSSV challenge [27]. The mRNA level of *SpJAK* was upregulated in response to exposure to MCRV, *V. parahaemolyticus*, *S. aureus*, Poly(I:C), and LPS [40]. The transcription level of *FcSTAT* increased corresponding to WSSV and *V. parahaemolyticus* challenge [45]. The enhanced expression of both *MjSTAT* and *MjSOCS* genes are observed following stimulation with peptidoglycan and polycytidylic acid [55]. The *SOCS* in the Chinese mitten crab was also upregulated after stimulation with Gram-positive bacteria [56]. In this study, the expression of *SpSTAT* was significantly responsive to challenge of MCRV, Poly(I:C), LPS and *S. aureus* (Fig. 7). After Poly(I:C) challenge, the expression of *SpSTAT* significantly changed in early challenge stage and reached a peak of 1.38-folf at 24 h

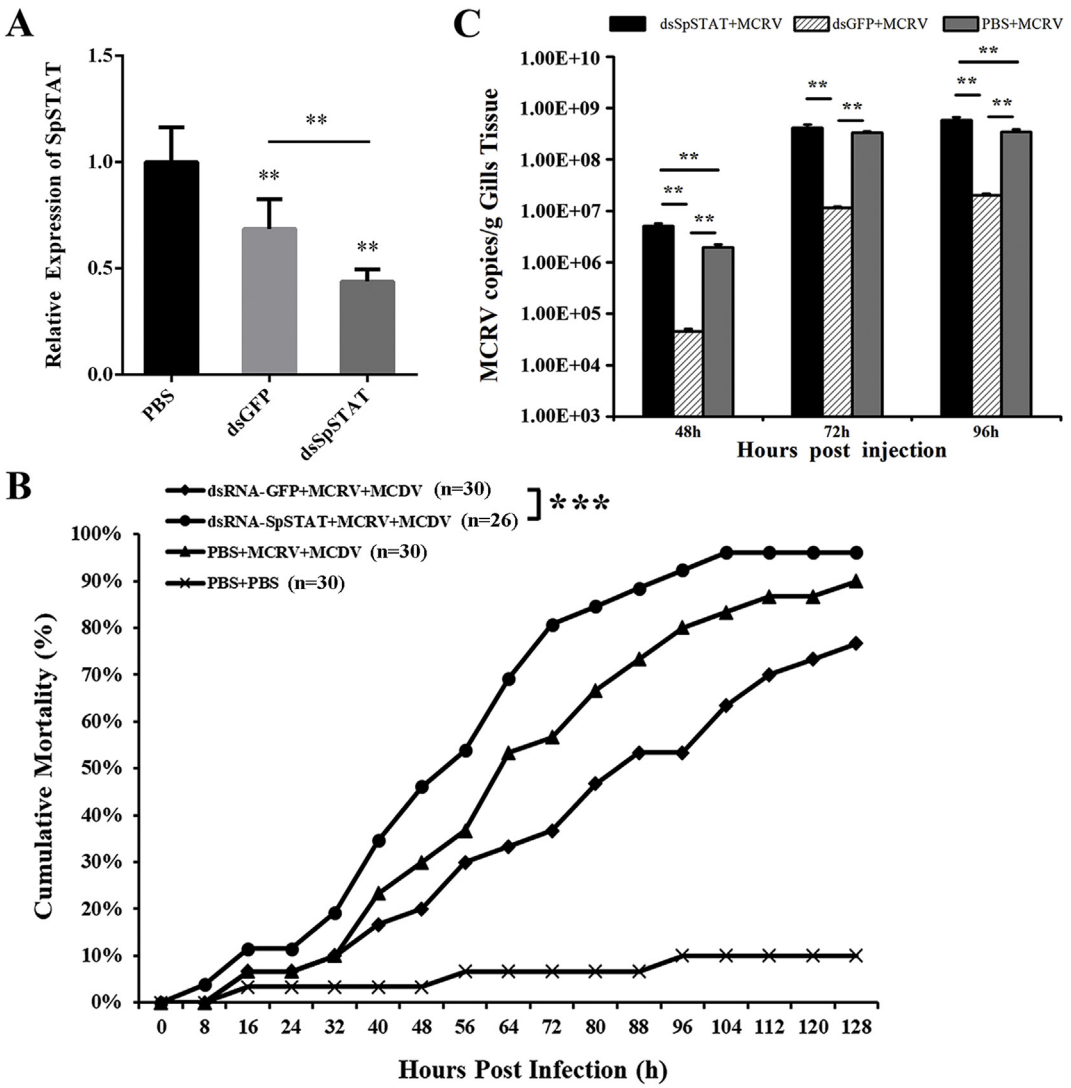

Fig. 8. Functional analysis of *Sp*STAT in MCRV infection. (A)Real-time PCR analysis of gene expression of *Sp*STAT at 48 h after injection with indicated dsRNA or PBS. The internal control was 18SrRNA (**$p < 0.01$); (B) Mortalities of *Sp*STAT-silencing mud crabs infected with MCRV. At 48 h after injection with indicated dsRNA or PBS, mud crabs were infected with MCRV and mock infected with PBS. Cumulative mortality was recorded every 8 h. Differences in cumulative mortalities between treatments were analyzed by *Kaplane*-Meier plot (log-rank X^2 test)(***$p < 0.001$); (C) MCRV genome copies in gill tissue (1 g) of *SpSTAT* dsRNA and control PBS and GFP dsRNA treated mud crabs at 48, 72 and 96 h post infection. Bars indicate the mean ± SD and statistical significances were calculated by the Student's t-test (**$p < 0.01$).

(Fig. 7B). Upon MCRV challenge, *SpSTAT* expression, similar with Poly (I:C) challenge, significantly changed in early 48 h and reached a peak of 1.76-folf at 36 h, then maintained the baseline expression level during 72–96 h (Fig. 7C). In response to LPS challenge, the expression level of *SpSTAT* obviously decreased at 24 h and obviously upregulated at 72 h and then slightly down-regulated at 96 h (Fig. 7D). During the *S. aureus* challenge, the mRNA level of *SpSTAT* early stage decreased to lower the baseline, whereafter, increased and reached a peak at 48 h, decreased to the lowest level at 96 h (Fig. 7E). Hence, *SpSTAT* plays a major role in responses to stimuli, which is consistent that *SpSTAT* could be activated by Poly(I:C) and LPS to translocate from the cytoplasm to the nucleus in S2 cell. In previous study, the mRNA level of *SpJAK* was upregulated in response to exposure to MCRV, *V. parahaemolyticus*, *S. aureus*, Poly (I:C), and LPS [40]. Hence, the JAK/STAT signaling pathway plays a major role in responses to immunostimulation in mud crab.

In the canonical mode of JAK-STAT signaling, STAT molecules are activated by JAK and dimers translocate to the nucleus, where they function as transcriptional activators, and bind to a specific palindromic DNA motif in the promoters of the JAK/STAT pathway target genes to activate transcription [4–6,57]. Genes regulated by the mammalian JAK-STAT pathway also include positive and negative regulators, which modulate the magnitude and/or duration of signaling [7–9]. The activated STAT proteins drive their own expression to form a positive feedback loop or compensate for activation-induced STAT degradation [7–9]. In insects, the core components of the fly JAK-STAT pathway function in a single linear manner that is typical of canonical JAK-STAT signaling. Moreover, the *Drosophila* JAK-STAT pathway is autoregulated by inducing positive and negative regulators [9]. As a positive regulator, STAT92E, similar to mammalian STATs [8], is transcriptionally induced by JAK-STAT signaling [10]. Mainly cytoplasmically localized in S2 cells, *Sp*STAT was not phosphorylated and activated to translocate to the nucleus by itself normally (Fig. 4A). After stimulated by poly(I:C) and LPS, *Sp*STAT was significantly activated to translocate to the nucleus in S2 cells (Fig. 4B). *Sp*STAT was activated to phosphorylate and translocate to the nucleus by *Sp*JAK (Fig. 5A). Combined with previous study, the mRNA level of *SpSTAT* was lower after injection of *SpJAK* dsRNA than injection of *SpSTAT* in the gills, and *Sp*JAK can activate the

promoter activity of the wsv069 gene and with the activities that significantly higher than that of the *Sp*STAT protein [40]. These results imply that *Sp*JAK is likely to promote the *Sp*STAT expression. Although expression of *SpSTAT* was responsive to immune-challenged, as a whole, there are not significant change of expression of *SpSTAT* after challenge of Poly(I:C) and LPS. These results are suggesting that *Sp*JAK, poly(I:C) and LPS could significantly activated *Sp*STAT to translocate to the nucleus in S2 cells, while, whether promote the expression of *Sp*STAT is worthy of further studies.

After poly(I:C) and LPS challenged, *Sp*STAT were largely translocate to the nucleus, but there are no band of *Sp*STAT phosphorylation. It may be that the being activated phosphorylation sites of *Sp*STAT are different between by *Sp*JAK and poly(I:C) and LPS, and phosphotyrosine antibody for *Lv*STAT only detected the phosphorylation site of being activated by *Sp*JAK. Tyrosine phosphorylation regulates the dimerization of STATs as an essential prerequisite for the establishment of a classical JAK-STAT signaling path, however, most vertebrate STATs contain a second phosphorylation site of serine phosphorylation [58], even though tyrosine phosphorylation sites are multiple in the transactivation domain of STAT5b [59,60]. Other studies have shown that unphosphorylated STAT proteins are constantly shuttling between cytoplasmic and nuclear compartments [61–63]. These are reasons why *Sp*STAT were largely translocate to the nucleus after stimulated by poly (I:C) and LPS, but no band of *Sp*STAT phosphorylation were detected, which is worthy of further studies.

In conclusion, a homologue of a signal transducers and activators of transcription gene from *S. paramamosain* was cloned and characterized, and it contains conserved protein domain of STATs. *Sp*STAT could be phosphorylated by interaction with *Sp*JAK and activated to translocate to the nucleus of S2 cells. Furthermore, *SpSTAT* expression was responsive to challenge of MCRV, Poly(I:C), LPS and *S. aureus*. In addition, *SpSTAT* could play antiviral roles against MCRV infection via activating the JAK/STAT signaling pathways.

Acknowledgment

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