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# Functional characterization of a protein inhibitor of activated STAT (PIAS) gene in *Litopenaeus vannamei*

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ABSTRACT

Protein inhibitor of activated STAT (PIAS) plays a critical role in the feedback modulation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway as a negative regulator in mammals and *Drosophila*, but the function of PIAS in crustaceans is still unclear. In this study, a PIAS termed *LvPIAS* was cloned and characterized from *Litopenaeus vannamei*. The full length of *LvPIAS* was 3065 bp, including a 2361 bp open reading frame (ORF) coding for a protein of 786 aa. *LvPIAS* expression was most abundant in muscle and could respond to the challenge of LPS, *Vibrio parahaemolyticus*, *Staphhylococcus aureus*, Poly I: C and white spot syndrome virus (WSSV). LvPIAS could be induced by the transcription factor LvSTAT, but LvPIAS could inhibit the transcriptional activity of LvSTAT to the LvPIAS promoter conversely, which indicated that there was a negative feedback loop between LvSTAT and LvPIAS. Furthermore, RNAi-mediated knockdown of *LvPIAS* shrimps showed higher survival rate to WSSV infection than those in the control group (dsGFP injection), suggesting that LvPIAS may play a negatively role against WSSV infection.

## 1. Introduction

The protein inhibitor of activated STAT (PIAS) family was initially identified as an inhibitor of STAT in the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway [1,2]. In mammals, the PIAS protein family consists of at least five members: PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy [3,4]. All the PIAS proteins have significant sequence similarity and highly conserved domains. They usually contain an N-terminal SAP box [including an Leu-Xaa-Xaa-Leu-Leu (LXXLL) signature motif], a highly conserved Pro-Ile-Asn-Ile-Thr (PINIT) motif, a highly conserved RING-finger-like zinc-binding domain (RLD), a highly acidic region (AD), and a C-terminal Ser/Thr amino acids enriched region (S/T) [5–8]. The PIAS proteins play an important role in a wide range of biological processes, including cell proliferation and differentiation, apoptosis, tumor development, and immune response [9–11].

In mammals and *Drosophila*, PIAS is involved in the negative regulation of the JAK/STAT signaling pathway by interacting with the activated STAT protein [12]. STAT is an important transcription factor in the JAK/STAT signaling pathway. STAT dimerizes in the cytoplasm and translocates into the nucleus where it combines with the corresponding cis-elements and triggers gene transcription. As the negative regulator of the JAK/STAT signaling pathway, PIAS can interact with the activated STAT and inhibit its translocation into the nucleus, resulting in the inhibition of STAT-mediated gene activation by blocking the DNA-binding activity of STAT [13,14]. As well as the regulation of STAT activity, PIAS also negatively regulates the Wnt [15], SMAD [16], and Nuclear factor-κB (NF-κB) [17] signaling pathways. In addition, PIAS has SUMO-E3-ligase activity, which mediates the binding of ubiquitin to the target protein [18].

Most studies of PIAS have focused on mammals and some model organisms, but the function of PIAS in aquatic organisms remains unclear [19]. In crustaceans, only two PIAS genes from *Scylla paramamosain* [19] and *Marsupenaeus japonicus* [20] were cloned and characterized. Pacific white shrimp, *Litopenaeus vannamei*, is the major economic shrimp species globally. In recent years, the shrimp farming industry has been challenged by various pathogens causing huge economic losses [21]. In L. *vannamei*, the JAK/STAT pathway plays an

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essential role in the innate antiviral response. LvDomeless [21], LvJAK [22] and LvSTAT [23] have been reported as three positive components while LvSOCS2 was considered as a negative regulator [24]. However, there are no reports about PIAS from *L. vannamei* until now.

In this study, a PIAS gene from *L. vannamei* (named *LvPIAS*) was cloned and characterized. In addition, *LvPIAS* expression in response to multiple stimuli and its functions in regulating the JAK/STAT pathway and during WSSV infection was investigated. These data on PIAS may help us better understand its function *in vivo* and the relationship between PIAS and the JAK/STAT pathway, which may help prevent shrimps from pathogenic infection.

## 2. Materials and methods

### 2.1. Biological materials

*L. vannamei* were collected from a shrimp farm in Zhanjiang city, Guangdong Province, China. They were cultured in a recirculating water tank system for over 7 days before experiments with water salinity and temperature maintained at 27‰ salinity at 25–27 °C, respectively. The shrimp were fed with a commercial shrimp pellet diet twice daily. White spot syndrome virus (WSSV) stock ($1 \times 10^5$ copies/mL) was prepared as previously described [25]. Gram-negative *Vibrio parahaemolyticus* and Gram-positive *Staphylococcus aureus* were cultured in Luria broth (LB) medium and finally adjusted to $1 \times 10^5$ CFU/mL, as described by Wang et al. [24].

### 2.2. Cloning of full length of LvPIAS cDNA

A partial EST sequence of *LvPIAS* were obtained from the *L. vannamei* transcriptome data. The full-length sequence of LvPIAS cDNA were amplified with the RACE method using the SMARTer™ RACE cDNA Amplification kit (Clontech, Japan) following the user manual and the specific primers were listed in Table 1. The specific products were purified by Gel Extraction Kit (Omega, USA), then cloned into the PMD-20 vector (TaKaRa, Japan) for sequencing.

### 2.3. Genome walking

The L. *vannamei* genome DNA was extracted from muscle tissues as we mentioned previously [25]. The promoter region of LvPIAS was isolated by Genome walking method. Genome walking libraries were constructed by GenomeWalker™ Universal Kit (Clontech, Japan) according to the manufacturer's protocol and the primers for performing the genome walking PCR amplification were listed in Table 1.

### 2.4. Sequence and phylogenetic analysis of LvPIAS

SMART program (http://smart.embl-heidelberg.de) was used to analyze the protein domain of LvPIAS. ExPASy Compute pI/Mw program (https://web.expasy.org/cgi-bin/compute_pi/pi_tool) was used to calculate the Theoretical *pI* and molecular mass (Mw). Amino acid sequences of PIAS homologues from other species were retrieved from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) databases and multiple sequence alignments were performed by ClustalW2 (http://www.ebi.ac.uk/tools/clustalw2). The neighbor-joining (NJ) phylogenic trees were constructed using the MEGA 6.0 software, and bootstrapping procedure with a minimum of 1000 bootstraps. The potential STAT transcription factor binding motifs in the promoter sequences of LvPIAS were analyzed with TRANSFAC@ 6.0 program [24] using high quality matrices and 0.80 as matrix and core similarity cut-off.

### 2.5. Subcellular localization analysis of LvPIAS

The expression vectors pAc5.1-N-GFP and pAc5.1-LvPIAS-GFP were constructed according to previous described method [26]. Drosophila S2 cells were used to analyze the subcellular localization of LvPIAS. For protein localization, Drosophila S2 cells were seeded onto the cover slips in 12-well plates (TPP, Switzerland) at 28 °C in a *Drosophila* serum-free medium (SDM; Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, USA). After 24 h, the cells were transfected with the pAc5.1-N-GFP and pAc5.1-LvPIAS-GFP using the Cellfectin II reagent (Invitrogen, USA). At 48 h post-transfection, cells on

**Table 1**
PCR primers used in this study.

| Primers | Primer sequences (5' -3') |
|---|---|
| **For cDNA cloning** | |
| LvPIAS-5′ RACE1 | AAGGGAGAAACTGAAGGTGGACT |
| LvPIAS-5′ RACE2 | CCTGAACGGAGTAGTCTTGTCT |
| LvPIAS-3′ RACE1 | GTGACCATCTCCAGTGTTTCG |
| LvPIAS-3′ RACE2 | ATGACTATCTGGTTATTGACGGCTAC |
| **For Genomic DNA cloning** | |
| 5′GW- LvPIAS-1[a] | GGGGTACCGCTATTCAGGTAGGTGGAGAGTTG |
| 5′GW- LvPIAS-2[a] | GGAGATCTAAGCAAATGCGACGAAGATCC |
| **For Protein expression** | |
| pAcLvPIAS-F[a] | GG**GGTACC**ATGCGGAGAATGGTTATGACCTTTCG |
| pAcLvPIAS-R[a] | GG**CTCGAG**CAGGAATCTGTCTTGTGGTCTGAAG |
| **For qPCR** | |
| qLvPIAS-F | TGTGTCCATTACCGACTCCAA |
| qLvPIAS-R | CGGCATAGGGCAGTCACATT |
| LvEF1α-F | GAAGTAGCCGCCCTGGTTG |
| LvEF1α-R | CGGTTAGCCTTGGGGTTGAG |
| **For RNAi** | |
| LvPIAS-F | GTGAATGTGACTGCCCTATGC |
| LvPIAS-R | GAAGTAGCCGTCAATAACCAGAT |
| LvPIAS-T7F[b] | GGATCCTAATACGACTCACTATAGGGTGAATGTGACTGCCCTATGC |
| LvPIAS-T7R[b] | GGATCCTAATACGACTCACTATAGGGAAGTAGCCGTCAATAACCAGAT |
| GFP-F | GTTCAGCGTGTCCGGCGAG |
| GFP-R | GTTCTTCTGCTTGTCGGCC |
| GFP-T7F[b] | GGATCCTAATACGACTCACTATAGGTCAGCGTGTCCGGCGAG |
| GFP-T7R[b] | GGATCCTAATACGACTCACTATAGGTCTTCTGCTTGTCGGCC |

[a] Nucleotides in bold indicate restriction sites introduced for cloning.
[b] T7 RNA polymerase promoter sequences are underlined.Figure legends.

the cover slips were washed three times with PBS and stained with Hoechst 33258 Solution (Beyotime, China). The treated cells were observed using a Leica confocal laser scanning microscope (Leica TCS-SP5, Germany).

### 2.6. Expression of LvPIAS in different tissues

The real-time quantitative polymerase chain reaction (qPCR) with the primers qLvPIAS-F and qLvPIAS-R (Table 1) was performed to detect the tissue distribution of *LvPIAS*.

LvEF-1α (GenBank accession No.GU136229) was used as control, with LvEF-1α-F and LvEF-1α-R as primers (Table 1). Eleven kinds of tissues, namely hemocyte, hepatopancrea, gill, intestine, epithelium, stomach, eyestalk, muscle, scape, pyloric ceca and heart were obtained from healthy *L. vannamei* for RNA extraction. Total RNA extraction, the reverse transcription and qPCR analysis were performed in detail as previous research [26]. qPCR assays were carried out with a Roche Light Cycler480 thermal cycler (Roche Applied Science, Germany). Expression level of LvPIAS was calculated using the Livak ($2^{-\triangle\triangle CT}$) method [27] after normalization to LvEF1α.

### 2.7. Immunity challenges and LvPIAS expression analysis

For immunity-challenged experiments, healthy shrimps (4–6 g each) were divided into six experimental groups, in which each *L. vannamei* was injected with 5 μg Lipopolysaccharide (LPS, Sigma), 5 μg Polyinosinic: polycytidylic acid (Poly I:C, Sigma), $1 \times 10^5$ colony-forming unit (CFU) of *S. aureus*, $1 \times 10^5$ CFU of *V. parahaemolyticus* or $1 \times 10^5$ copies of WSSV particles in 50 μL PBS, respectively [24]. The negative control group received an injection of 50 μL PBS only. The gills of challenged shrimps were sampled at 0, 4, 8, 12, 24, 36, 48, 72 h post injection (hpi), and three samples from each group were randomly selected. The relative mRNA expression of LvPIAS was detected by qPCR using the same program described above.

### 2.8. Dual-luciferase reporter assays

The promoter of LvPIAS was cloned into the firefly luciferase plasmid pGL3-Basic (Promega, USA) to generate the reporter plasmid named as PGL3-LvPIAS. The ORFs of LvSTAT and LvPIAS genes were cloned into pAc5.1/V5-His A plasmid (Invitrogen, USA) to generate their expression vectors named as pAc5.1-LvSTAT and pAc5.1-LvPIAS, respectively. For dual luciferase reporter assays, the *Drosophila* S2 cells were plated in 96-well plate and transfected with 0.03 μg of reporter gene plasmids (PGL3-LvPIAS), 0.03 μg of pRL-TK renilla luciferase plasmids (as an internal control), and a gradient of (0.03, 0.05 and 0.1 μg, respectively) of pAc5.1-LvSTAT. In addition, to explore the effect of LvPIAS on the transcriptional activity of LvSTAT, a gradient of (0.03, 0.05 and 0.1 μg, respectively) of pAc5.1-LvPIAS and 0.1 μg of pAc5.1-LvSTAT were also co-transfected to S2 cells, with 0.03 μg of reporter gene plasmids (PGL3-LvPIAS) and 0.03 μg of pRL-TK renilla luciferase plasmids (as an internal control). Cells were harvested at 48 h post transfection and lysed for examination of firefly and renilla luciferase activities using a Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacture's protocol. Each experiment was performed at least three times.

### 2.9. Knockdown of LvPIAS expression by dsRNA-mediated RNA interference

The double-stranded RNAs (dsRNAs) were synthesized using an in vitro transcription method by using the T7 RiboMAX™ Express RNAi System (Promega, USA) according to the user manual as described in our previous studies [25], and the quality and amount of dsRNAs were checked using 1% agarose gel electrophoresis and a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Primers used for the synthesis of dsRNAs were shown in Table 1. The length of LvPIAS and GFP dsRNAs were 479 bp and 554 bp, respectively. The experimental group was injected with dsRNA-LvPIAS (2 μg/g shrimp), while the control groups were injected with equivalent dsRNA-GFP. RNA interference efficiency was investigated using qPCR. Gill samples were taken from 9 shrimps in each challenge group at 48 h after injection, and 3 shrimps were pooled together. Total RNAs was extracted and reverse transcribed into cDNA for qPCR. The LvEF1-α was used as the internal control. Primer sequences were listed in Table 1.

### 2.10. WSSV challenge experiments in LvPIAS-knockdown shrimps

Healthy L. *vannamei* (average 4–6 g, n = 40) received an intramuscular injection of dsRNA-LvPIAS or dsRNA-GFP (2 μg/g shrimp). Forty-eight hours later, shrimps were injected again with $1 \times 10^5$ copies of WSSV particles and mock-challenged with PBS as a control. The shrimps were cultured in tanks with air-pumped circulating seawater and were fed with artificial diet three times a day at 5% of body weight for about 8 days following infection. The mortalities of each group were counted every 4 h and the differences between groups were analyzed by using the Mantel-Cox (log-rank $\chi^2$ test) method with the GraphPad Prism software.

### 2.11. Statistical analysis

All numerical data were presented as the mean ± standard deviation. The means of two samples were compared by Student's t-test. The differences were significant at $p < 0.05$ in all cases. All experiments were repeated at least three times. Differences in mortality levels between treatments were analyzed by Kaplan–Meier plot (log-rank $\chi^2$ test).

## 3. Results

### 3.1. cDNA characterization of LvPIAS

The full-length cDNA sequence of LvPIAS was obtained. The cDNA was 3065 bp in length and was comprised of a 259-bp 5′-untranslated region (UTR), a 445-bp 3′-UTR containing a poly(A) tail, and an open reading frame (ORF) of 2361 bp that encoded a protein of 786 aa (GenBank accession number: MK987066; Fig. 1A). Using the ExPASy Compute pI/Mw program, the theoretical *pI* and molecular mass (Mw) of the deduced protein were predicted as 8.02 and 83.9 kDa, respectively. As described by Wang et al. [28], structure prediction by SMART showed that the LvPIAS protein contained four typical domains (Fig. 1B), including a SAP domain (12–46 aa) with an LXXLL motif, a PINIT superfamily domain (156–307 aa), an RLD domain (352-401aa), and a serine/threonine-rich repeat C-terminal domain (585–733 aa).

### 3.2. Phylogenetic analysis of LvPIAS

The alignment of the LvPIAS amino acid sequence by BLASTP showed high identity (99%) with *M. japonicus* PIAS and 31–86% with those of other invertebrates and vertebrates(Fig. 2). Based on the BLASTP results, a phylogenetic tree was constructed by MEGA 6.0 with the neighbor-joining method to study the evolutionary relationship among them. As shown in Fig. 3, LvPIAS was placed into the group of Arthropoda, and was closest to MjPIAS (*M. japonicus* PIAS, AZB50217.1) and SpPIAS (*S. paramamosain* PIAS, AHH29321.1).

### 3.3. Subcellular localization analysis

Analysis of the subcellular localization is important for in-depth studies of protein biological functions such as the signal transduction and interaction. We inserted the full-length ORF of LvPIAS into the pAc-GFP vector to visualize the subcellular localization by green

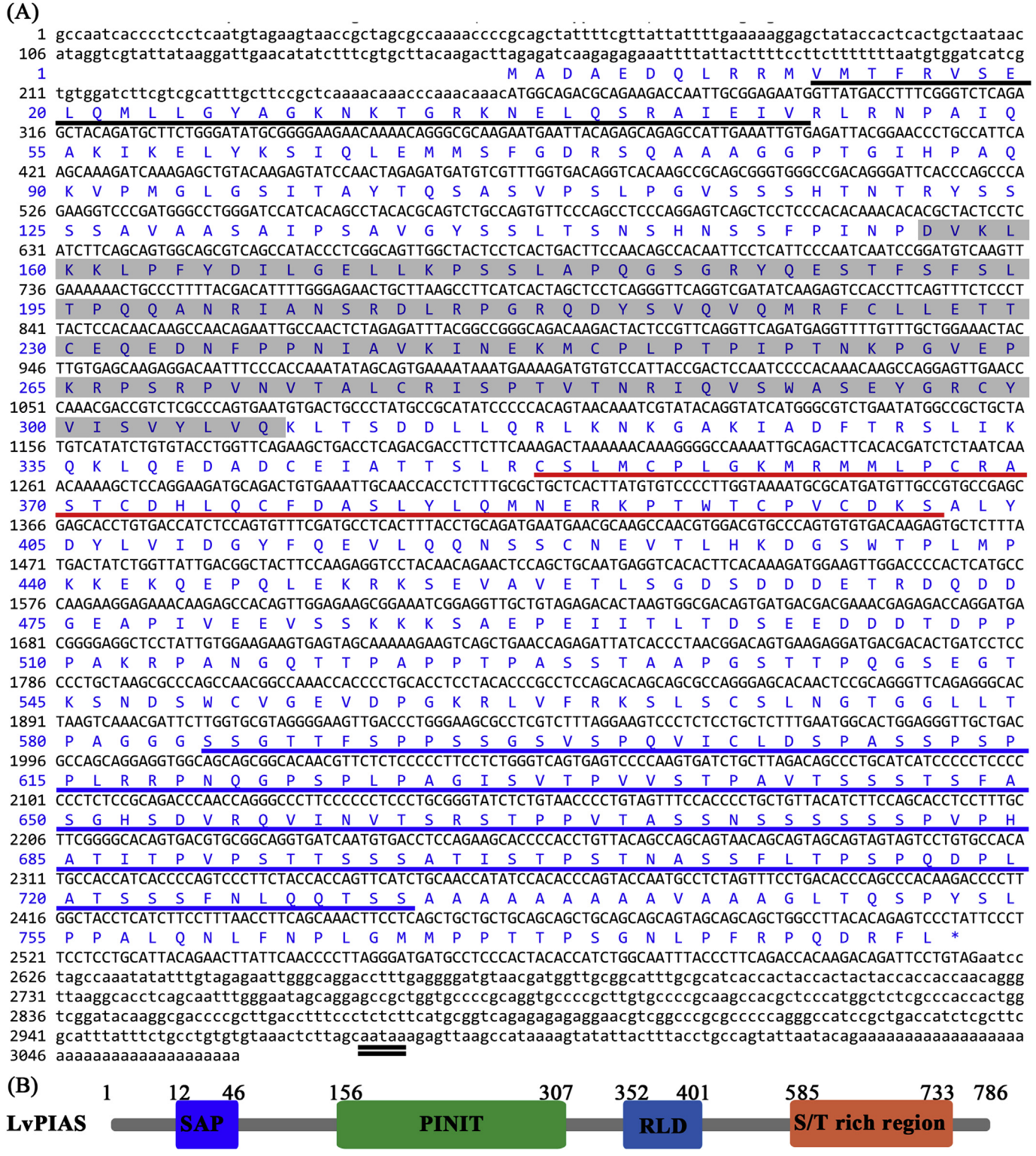


**Fig. 1.** The sequence analysis of LvPIAS. (A) The full-length cDNA sequence and deduced amino acid sequences of LvPIAS. The ORF of the nucleotide sequence is shown in upper-case letters, while the 5′ and 3′-UTR sequences are shown in lowercase. Amino acid sequence is represented with one-letter codes above the nucleotide sequence. The putative SAP domain, RLD domain and S/T rich repeat domain were underlined in black, red and blue, respectively. The putative PINIT domain was shown in shadow, as well as the Poly (A) addition signals (aataaa) is denoted by a double underline.
(B) Domain diagrams of LvPIAS. Region shaded in blue indicated SAP domain, green indicated PINIT domain, pale blue indicated RLD domain and orange indicated S/T rich repeat domain, respectively. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

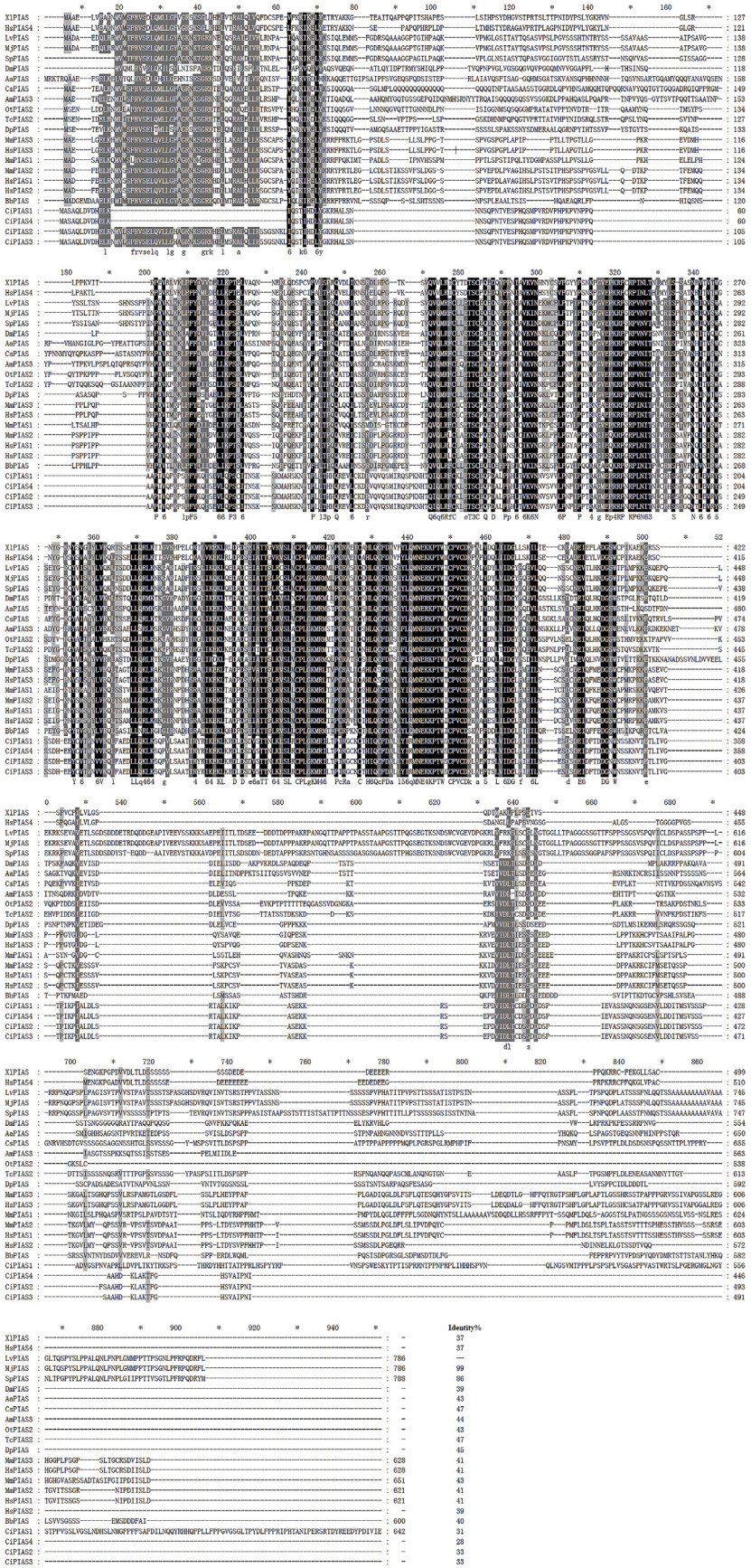


**Fig. 2.** Multiple alignment of PIAS proteins from different species.The amino acid sequences of PIAS from typical organisms were aligned using the ClustalX2.0 program (http://www.ebi.ac.uk/tools/clustalw2).The Genbank accession numbers of the aligned PIAS sequences are listed in the legend of Fig. 3.

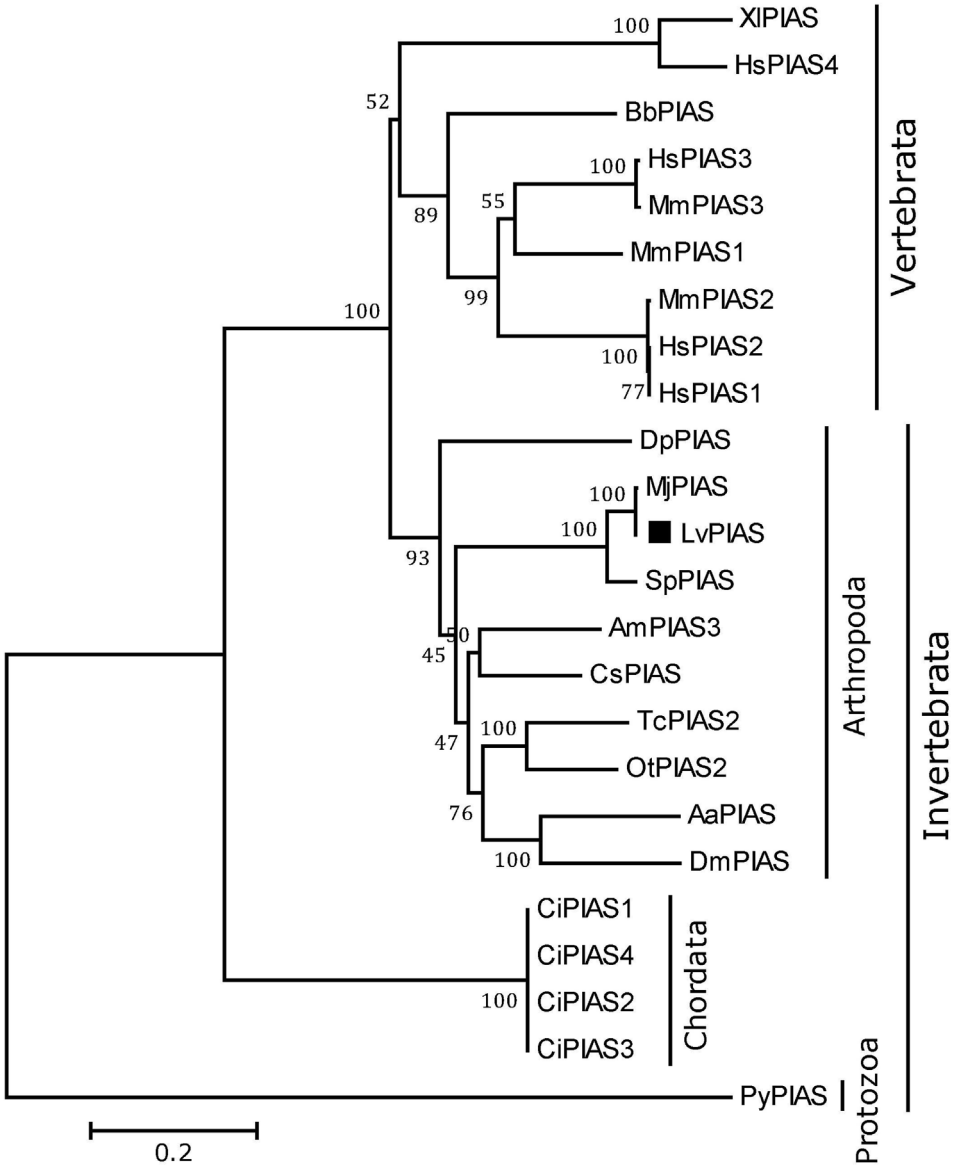


**Fig. 3.** Phylogenetic analysis of LvPIAS. The deduced amino acid sequence of LvPIAS was aligned with other known PIASs by the ClustalW program and the tree is constructed using MEGA 6.0 software with neighbor-joining method. The bootstrap sampling is performed with 1000 replicates. LvPIAS was marked by ■ HsPIAS1, *Homo sapiens* PIAS1 (Accession No. NP_004662.2); HsPIAS2, *H. sapiens* PIAS2 (Accession No. NP_775298.1); HsPIAS3, *H. sapiens* PIAS3 (Accession No. NP_006090.2); HsPIAS4, *H. sapiens* PIAS4 (Accession No. NP_056981.2); MmPIAS1, *Mus musculus* PIAS1 (Accession No. NP_062637.2); MmPIAS2, *M. musculus* PIAS2 (Accession No. NP_032628.3); MmPIAS3, *M. musculus* PIAS3 (Accession No. NP_001159421.1); BbPIAS, *Branchiostoma belcheri* PIAS (Accession No. ARV76530.1); XlPIAS, *Xenopus laevis* PIAS (Accession No. NP_001082751.1); MjPIAS, *M. japonicus* PIAS (Accession No. AZB50217.1); SpPIAS, *S. paramamosain* PIAS, (Accession No. AHH29321.1); DpPIAS, *Daphnia pulex* PIAS, (Accession No. EFX76934.1); AmPIAS, *Apis mellifera* PIAS, (Accession No. XP_623571.3); CsPIAS, *Cryptotermes secundus* PIAS, (Accession No. XP_023712328.1); TcPIAS, *Tribolium castaneum* PIAS, (Accession No. XP_974023.2); OtPIAS, *Onthophagus taurus* PIAS, (Accession No. XP_022913976.1); AaPIAS, *Anopheles aquasalis* PIAS, (Accession No. AEK26394.1); DmPIAS, *Drosophila melanogaster* PIAS, (Accession No. NP_724750.1); CiPIAS1, *Ciona intestinalis* PIAS1, (Accession No. XP_009861714.1); CiPIAS2, *C. intestinalis* PIAS2, (Accession No. XP_009861715.1); CiPIAS3, *C. intestinalis* PIAS3, (Accession No. XP_009861716.1); CiPIAS4, *C. intestinalis* PIAS4, (Accession No. XP_009861717.1); PyPIAS, *Plasmodium yoelii* PIAS, (Accession No. CDU19070.1).

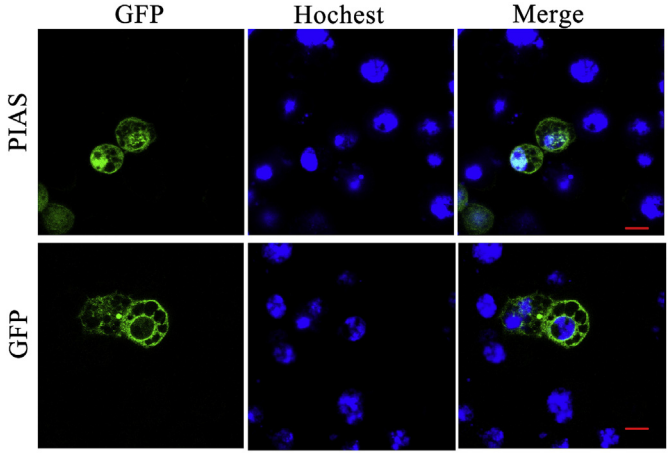


**Fig. 4.** Subcellular localization of LvPIAS. Drosophila S2 cells were transfected with plasmids pAc5.1-LvPIAS-GFP or pAc5.1-GFP (as a control). At 36 h post-transfection, the cells were observed using a Leica confocal laser scanning microscope.

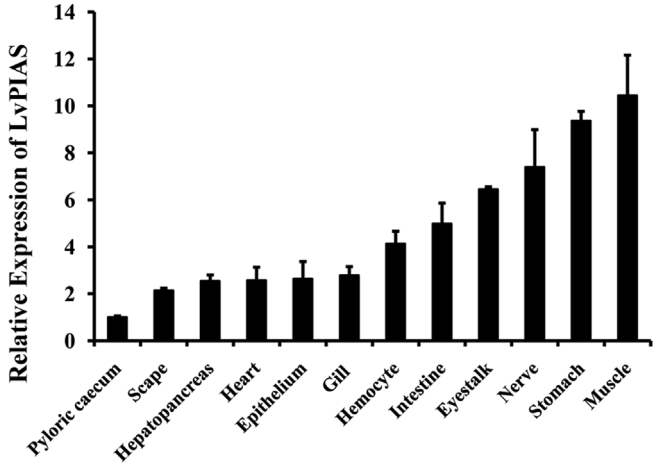


**Fig. 5.** Tissue distribution of *LvPIAS* expression in healthy *L. vannamei.* Ten animals were used for tissue sampling. LvEF-1a was used as the internal control to normalize the cDNA template used for qPCR analysis. The expressions of LvPIAS in pyloric caecum was set as 1.0. The results were based on three independent experiments and expressed as mean values ± SD.

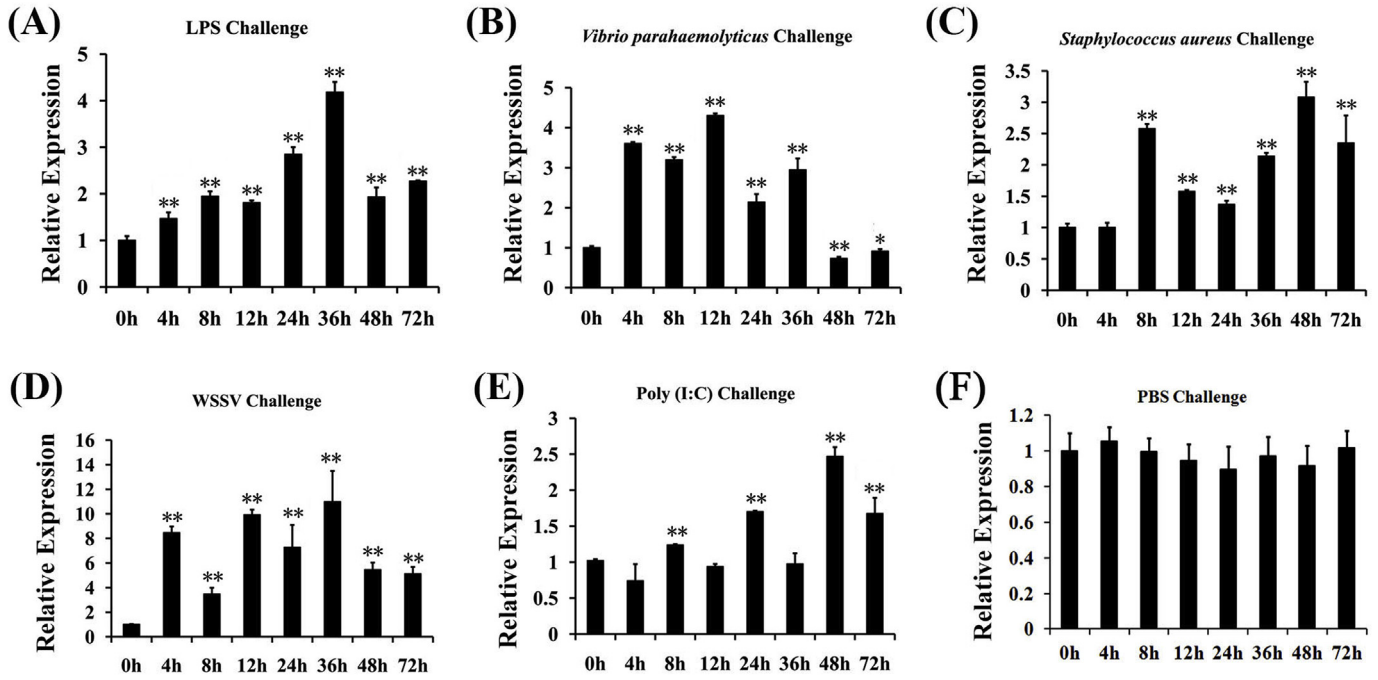


**Fig. 6.** Expression profiles of *LvPIAS* in gill from pathogens or stimulants challenged *L. vannamei.* qPCR was performed in triplicate for each sample. Expression values were normalized to those of EF-1α using the Livak ($2^{-\triangle\triangle CT}$) method. Results plotted as mean ± SD of triplicate assays (**$p < 0.01$, *$p < 0.05$).

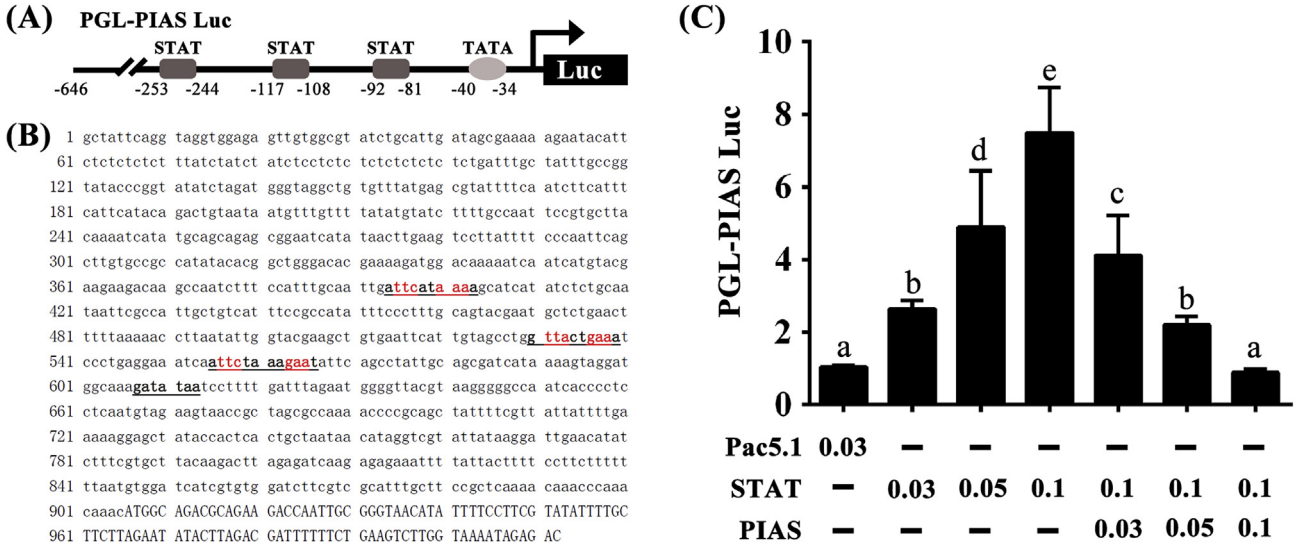


**Fig. 7.** The dual-reporter assay of the promoter of LvPIAS. (A) Schematic representation of the architecture of PGL3-LvPIAS. The PGL3-LvPIAS plasmid contains 3 potential STAT binding motifs and a TATA box, followed by a firefly luciferase gene (LUC). (B) The promoter sequence of LvPIAS. The promoter sequences are shown in lower-case letters and the coding sequences are shown in uppercase. The predicted TATA-box was boxed and the putative STAT binding sites were underlined. (C) Effects of LvSTAT or (and) LvPIAS on the activity of LvPIAS promoter in *Drosophila* S2 cells. Values (mean ± SD) in bars that have the same letter are not significantly different ($p > 0.05$; Tukey's test) of the luciferase activities among treatments (n = 3).

fluorescence using confocal laser scanning microscopy. As shown in Fig. 4, the recombinant GFP-tagged LvPIAS was expressed in *Drosophila* S2 cells and could be observed in both the cytoplasm and the nucleus.

### 3.4. Tissue distribution of LvPIAS in healthy L. vannamei

qPCR was performed to determine the expression levels of *LvPIAS* in different tissues. Transcripts of *LvPIAS* could be detected in all the examined tissues (Fig. 5). The highest expression of *LvPIAS* was detected in muscle and the lowest expression was in the pyloric caecum; the expression level of *LvPIAS* in muscle was nearly 11 times that in the pyloric caecum. In addition, *LvPIAS* showed high expression in the stomach, nerves, and eyestalk. Tissues involved in immune defense, *i.e.*, hemocyte, gill, and hepatopancrea, had moderate levels of *LvPIAS* expression.

### 3.5. Expression of LvPIAS in immune-challenged L. vannamei

Several stimuli were applied in the immune challenge experiments and the temporal expression of *LvPIAS* was measured by qPCR. The gill was chosen to delineate the time-course expression changes. After LPS injection, the *LvPIAS* mRNA level increased and peaked at 36 h, after which the mRNA level slightly decreased at 48–96 h, but the expression level at 48 h and 96 h still exceeded that of 0 h (Fig. 6A). After *V.*

*parahaemolyticus* challenge, the transcriptional levels of *LvPIAS* were upregulated at 4–36 h and reached a peak of ~4.31-fold at 12 h, and then subsequently decreased and returned to the baseline level at 72 h (Fig. 6B). After *S. aureus* challenge, the expression level of *LvPIAS* showed no change at 4 h, then increased rapidly at 8 h and maintained a high level during the remainder of the time course (Fig. 6C). In response to WSSV treatment, the transcript levels of *LvPIAS* increased by ~8.4-fold at 4 h, then slightly decreased at 8 h, followed by another increase from 12 h to 36 h with a peak of ~11.0-fold at 36 h, and decreased and maintained a constant expression profile at 48–72 h (Fig. 6D). After poly I:C challenge, *LvPIAS* expression was slightly increased at 8 h, then returned to the baseline at 12 h, followed by another increase at 24 h, after which it maintained a high level at 48–72 h (Fig. 6E). In the PBS-treated group, the transcriptional levels of *LvPIAS* did not significantly change throughout the time course (Fig. 6F).

### 3.6. LvSTAT induces the activity of LvPIAS promoter

A 2000-bp sequence upstream of the transcriptional initiation site (TSS) of *LvPIAS* was amplified using the genome-walking method. Using the TRANSFAC program, three potential STAT-binding motifs in the promoter of *LvPIAS* were predicted (Fig. 7A and B), suggesting that the expression of *LvPIAS* could be regulated by LvSTAT. To investigate whether LvSTAT could enhance the activity of the *LvPIAS* promoter, dual-luciferase reporter assays were performed. As shown in Fig. 7C, overexpression of LvSTAT in *Drosophila* S2 cells increased the activity of the *LvPIAS* promoter, and the activity of LvPIAS promoter could be induced gradually with the increasing overexpression levels of LvSTAT. Together, these data indicate that *LvPIAS* is a transcriptional target gene of LvSTAT.

### 3.7. LvPIAS inhibits the transcriptional activity of LvSTAT

To analyze whether LvPIAS could inhibit the transcriptional activity of LvSTAT, a concentration gradient of LvPIAS expression plasmids were co-transfected into *Drosophila* S2 cells with 0.1 μg of pAc5.1-LvSTAT, and the activity of the *LvPIAS* promoter was examined by dual-luciferase reporter assays. As shown in Fig. 7C, LvPIAS inhibited the transcriptional activity of LvSTAT, and the luciferase activity gradually decreased with the increasing levels of transfected pAc5.1-LvPIAS, suggesting that there could be an interaction between LvPIAS and LvSTAT.

### 3.8. Knockdown of LvPIAS decreased the mortality of WSSV-infected L. vannamei

To determine the function of *LvPIAS* in response to viral invaders, RNA interference (RNAi) was used to suppress *LvPIAS* expression. The silencing efficiency was checked using qPCR. As shown in Fig. 8A, the mRNA level of *LvPIAS* was remarkably downregulated in dsRNA-LvPIAS-treated shrimps, whereas there was no suppressive effect on *LvPIAS* in the dsRNA-GFP-treated group.

Experimental shrimps were challenged with WSSV at 2 days post dsRNA. As shown in Fig. 8B, during WSSV infection, the dsRNA-LvPIAS group showed a much slower increase in cumulative mortality than that of the dsRNA-GFP group (Kaplane Meier log-rank $\chi^2$: 6.891, $p < 0.01$). In the WSSV-challenged group, the cumulative mortality in the dsRNA-GFP-treated group was 64.7% while that in the LvPIAS knockdown group was only 31.3%.

## 4. Discussion

The JAK/STAT pathway is one of the important immune signaling pathways and exists widely from fruit flies to humans [29]. Several negative feedback regulators of JAK/STAT pathway such as SOCS (suppressors of cytokine signaling), PIAS and PTPs (protein tyrosine phosphatases) have been found in shrimp [20,30,31]. However, PIAS has not been reported in *L. vannamei*. In this study, a PIAS gene was cloned and characterized from *L. vannamei*. It was found that there may be a negative feedback loop between LvSTAT and LvPIAS. To the best of our knowledge, this is the first report of a negative feedback loop between PIAS and STAT of the JAK/STAT pathway in invertebrates.

Like other PIAS proteins, LvPIAS contains several typical conserved domains, including the SAP, PINIT, RLD, and S/T domains. These domains have different functions. For example, the SAP domain is involved in sequence- or structure-specific DNA binding [32], while the RLD domain is required for the SUMO-E3-ligase activity of PIAS proteins and might be involved in the interaction with other proteins [33]. Sequence alignment results showed that LvPIAS displayed 99.11% identity with the amino acid sequence of MjPIAS from *M. japonicus*, indicating that they may have similar biological functions. The subcellular localization of LvPIAS was investigated and LvPIAS could be observed in the cytoplasm. Previous studies showed that PIAS could bind to the transcription factor STAT in the cytoplasm and then inhibit the DNA-binding activity and activity of downstream genes [20]. LvPIAS was located in the cytoplasm, suggesting that LvPIAS may play a similar role as PIAS proteins from other species. Meanwhile, LvPIAS could also be observed in the nucleus, suggesting that LvPIAS could also

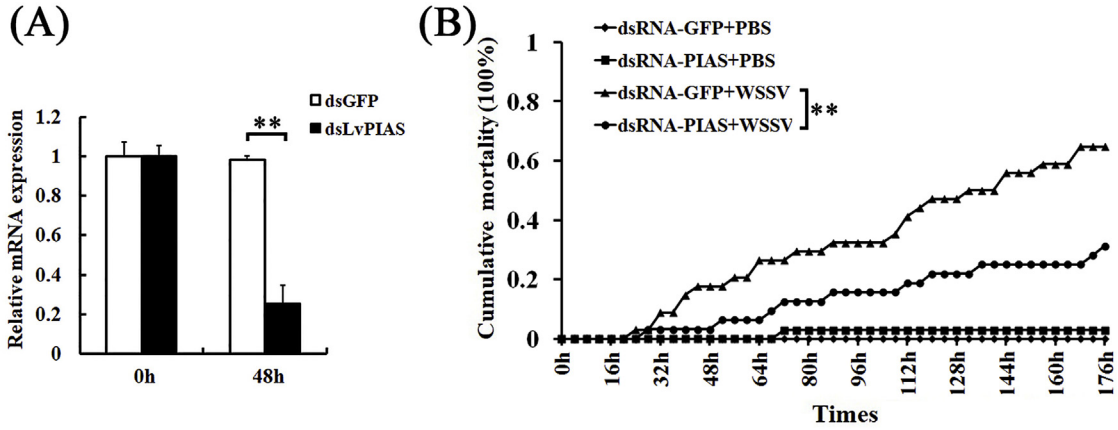


**Fig. 8.** Function of LvPIAS during WSSV infection. (A) qPCR analysis of the silencing efficiencies of LvPIAS, the internal control was EF-1α. Samples were taken at 48 h after injection with indicated dsRNA; (B) Shrimps were injected intramuscularly with dsRNA-LvPIAS or dsRNA-GFP. At 48 h after the initial injection, shrimps were infected with WSSV. Cumulative mortality was recorded every 4 h. Statistical significances between dsRNA-LvPIAS and dsRNA-GFP groups were calculated using the log-rank $\chi^2$ test (**$p < 0.01$ and *$p < 0.05$).

interact with activated STAT in the nucleus.

As a member of the negative regulators of the JAK/STAT signaling pathway, the PIAS protein participates in the regulation of not only cytokines but also immune signaling pathways [9]. Our expression analysis indicated that *LvPIAS* mRNA was widely expressed in all the examined tissues, including the hemocyte, gill, and hepatopancrea, which act as the main immune tissues in shrimp, suggesting that LvPIAS might also participate in the immune response *in vivo* similar to other PIAS proteins. We also explored the response to various stimuli of LvPIAS in gill, including LPS, poly I:C, *V. parahaemolyticus*, *S. aureus,* and WSSV. After the LPS, *V. parahaemolyticus* and *S. aureus* challenges, the *LvPIAS* expression was significantly upregulated from 4 to 36 h, which suggests that LvPIAS might be associated with antibacterial immunity. Similar results were obtained in MjPIAS in which the *MjPIAS* expression was upregulated from 6 to 48 h post *V. anguillarum* infection [20]. In addition, after poly I:C and WSSV challenge, the mRNA level of *LvPIAS* increased significantly, especially with WSSV infection. The *LvPIAS* expression was significantly upregulated from 4 to 72 h and reached a peak at 36 h, which was several-folds higher than that of other stimuli, suggesting that LvPIAS might also be involved in antiviral immunity.

Although some studies have shown that STAT could be exploited by WSSV to enhance viral replication and infection in shrimp [23,34], more studies showed that the JAK/STAT signaling pathway plays a positive role in antiviral immunity and STAT is involved in the antiviral immune defense as an important transcription factor [35]. In *M. japonicus* [36]and *M. rosenbergii* [37], the number of WSSV copies in shrimp was both significantly increased compared with the control when the expression of *MjSTAT* and *MrSTAT* were knocked down, respectively. Meanwhile, MjPIAS can negatively regulate the JAK/STAT pathway by inhibiting STAT phosphorylation and translocation and inhibited the expression of several antimicrobial peptides (AMPs). Knockdown of MjPIAS enhanced STAT phosphorylation and translocation, resulting in increased bacterial clearance and survival rate [20]. In this study, shrimps treated with RNAi-mediated knockdown of LvPIAS showed lower susceptibility to WSSV infection, in other words, suppression of LvPIAS meant the hyperactivated JAK/STAT pathway activity with stronger antiviral ability, indicating that LvPIAS also negatively regulates antiviral immunity activity of the JAK/STAT pathway like LvSOCS2. Considering the high sequence similarity with MjPIAS, we speculate that knockdown of LvPIAS suppressed its inhibitory activity to the activated STAT and then a large number of activated STAT proteins subsequently translocated into the nucleus and activated the expression of antiviral genes, eventually eliciting a global antiviral immune response in shrimp. In fact, STAT could regulate the expression of several AMPs and thioester-containing proteins (TEPs) in shrimp, in which LvSWD4 (AMPs) [38], MjTEP1, and MjTEP2 (TEPs) [36] exhibited not only antibacterial but also antiviral activity.

As well as STAT being involved in the regulation of expression of AMPs and TEPs, STAT can also regulate the expression of core members of the JAK/STAT signal pathway in shrimp, such as JAK and SOCS2, therefore creating feedback regulatory loops. In L. *vannamei*, LvJAK is induced by itself and LvSTAT and positively regulates the response of the JAK/STAT pathway, indicating that there is a positive feedback loop between LvJAK and LvSTAT [22]. Similarly, LvSOCS2 is also induced by LvSTAT and forms a negative feedback loop in the JAK/STAT pathway [24]. In this study, we found that LvSTAT positively regulated the expression of LvPIAS. Meanwhile, LvPIAS acted as a negative regulator of the JAK/STAT pathway and impressed the transcriptional activity of LvSTAT similar to other PIAS proteins, suggesting there is also a negative feedback loop between LvSTAT and LvPIAS. These results indicate that these core members of the JAK/STAT pathway are form a complicated regulatory network in shrimp, and together maintains the dynamic balance of the immune response. On the one hand, these positive regulators regulate the expression of downstream genes to protect against pathogen invasion while, on the other hand, negative regulators limited the over-reaction of the immune response to protect the host from damage.

In conclusion, a shrimp PIAS from *L. vannamei* was cloned and characterized. The results indicate that LvPIAS is a negative regulator of the JAK/STAT pathway. In addition, there may be a negative feedback loop between LvPIAS and LvSTAT in *L. vannamei*. Further studies on the interaction between LvPIAS and LvSTAT will reveal the mechanism of the PIAS-mediated antiviral defense of the JAK/STAT pathway.

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