Fish and Shellfish Immunology 89 (2019) 555–563

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

A JAK-STAT pathway target gene encoding a single WAP domain (SWD)-containing protein from *Litopenaeus vannamei*

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ARTICLE INFO

Keywords:
JAK-STAT pathway
Single WAP domain-containing peptide
Antibacterial activity
Immune responses
Litopenaeus vannamei

ABSTRACT

In shrimp, the JAK-STAT pathway is essentially implicated in both antiviral and antibacterial responses. However, few regulatory target genes of the JAK-STAT pathway in shrimp have been reported so far. In this study, a novel single WAP domain-containing peptide (LvSWD4) was identified from Pacific white shrimp *Litopenaeus vannamei*. The promoter of LvSWD4 was predicted to harbor multiple STAT-binding DNA motifs. Over-expression of the JAK-STAT pathway components STAT, JAK and Domeless *in vitro* significantly enhanced the transcriptional activity of the LvSWD4 promoter, and *in vivo* silencing of STAT and the the JAK-STAT pathway upstream regulator IRF down-regulated the expression of LvSWD4, suggesting that LvSWD4 could be a target gene of the JAK-STAT pathway. The expression of LvSWD4 was significantly increased after infection with Gram-negative and positive bacteria, fungi and virus, and silencing of LvSWD4 increased the susceptibility of shrimp to *V. parahaemolyticus* and WSSV infections. *In vitro* experiments also demonstrated that the recombinant LvSWD4 protein had significant inhibitory activities against Gram negative bacteria *V. parahaemolyticus* and *E. coli* and Gram positive bacteria *S. aureus* and *B. subtilis*. Furthermore, silencing of LvSWD4 *in vivo* significantly affected expression of various immune functional genes and attenuated the phagocytic activity of hemocytes. These suggested that as a target gene of STAT, LvSWD4 was essentially implicated in shrimp immunity, which could constitute part of the mechanism underlying the immune function of the shrimp JAK-STAT pathway.

1. Introduction

Signal transducer and activator of transcription (STAT) is a family of conserved transcription factors widely present in metazoans, activation of which normally depends on the non-receptor Janus kinase (JAK)-mediated tyrosine phosphorylation [1]. The JAK/STAT pathway embracing a diversity of downstream target genes is a pivotal part of the innate immune system. In mammals, the JAK/STAT pathway serves as a key intracellular signal transduction channel downstream of various cytokines, interleukins and growth factors [2]. Through the JAK/STAT pathway, interferons (IFNs) produced in response to viral infection activate a series of interferon stimulated genes (ISGs) to establish the cellular antiviral state [3]. Although lacking the real IFN system, invertebrates possess the JAK/STAT pathway, activation of which is also central to the antiviral responses [4,5]. Compared with that in mammals, the JAK/STAT pathway in invertebrates is relatively simple with fewer components. In *Drosophila*, the fundamental structure of the JAK/STAT pathway consists of three unpaired ligands (*Upd1*, *Upd2* and *Upd3*), a Domeless receptor, a JAK kinase Hopscotch (*hop*), a STAT called STAT92E and a set of STAT-target genes, such as *zfh2*, *raf*, *tep-1* and *Socs36e* [5–8].

The Crustin family is a group of antimicrobial peptides (AMPs) that play important roles in defense against a spectrum of invading pathogens in invertebrates [9]. Crustins contain a C-terminal whey acidic protein (WAP) domain of ~50 amino acids that possesses a four-disulfide core (4-DSC) consisting of eight conservatively arranged cysteines [10]. The crustin family can be categorized into I, II and III subgroups according to the domain structure between the WAP domain and the N-terminal signal sequence [11,12]. Type I and II crustins were characterized by the Cys-rich, and Gly/Cys-rich regions in the C-terminal regions, respectively, while type III crustins have only one WAP domain and are also named single WAP domain-containing peptides (SWDs) [13,14].

The Pacific white shrimp, *Litopenaeus vannamei* is a representative

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https://doi.org/10.1016/j.fsi.2019.04.046
Received 6 January 2019; Received in revised form 11 April 2019; Accepted 12 April 2019
Available online 15 April 2019
1050-4648/

Table 1
Summary of primers used in this study.

Primers	Sequences(5′ to 3′)
DNA cloning	
LvSWD4-ORF-F	ATGATGACTTTTCGAGGAATAAGTGTGTG
LvSWD4-ORF-R	GGATCGGATGAGGAGATGAGGAGA
LvSWD4-5′RACE1	TCAACGCAAGATAACCCACC
LvSWD4-5′RACE2	TCAACTTCCTGTTCGGTGCT
LvSWD4-3′RACE1	GCGTCAAGAATGTGGAGTGC
LvSWD4-3′RACE2	GCAGGATTCGGATTGTGATG
Real-time PCR analysis	
LvSWD4-F	ACGAGCACCGAACAGGAAG
LvSWD4-R	CGCAAGATAACCCACCAGG
Lv*EF-1a*-F	CCTATGTGCGTGGAGACCTTC
Lv*EF-1a*-R	GCCAGATTGATCCTTCTTGTTGAC
LvSTAT-F	CTTCGCCATCCGTCCTCTAG
LvSTAT-R	GGCTTGATCCTTAGGCACATTC
LvIRF-F	GCGAATGGCCCTGAATAAGT
LvIRF-R	GCGGCGATGTCGTAGGAAT
LvPEN2-F	CCAAGGCGAAGCGTACAG
LvPEN2-R	CAATTGCGAGCATCTGAGAC
LvPEN3-F	CTCCTGCGTCCGCCATG
LvPEN3-R	GTGTAACCGCCCTTGTACAC
LvPEN4-F	GCCCGTTACCCAAACCATC
LvPEN4-R	AACAATCCCCGTATCTGAAGC
LvCTL2-F	ACAAGCGGAGCAGTTCTGG
LvCTL2-R	CAGTCACCTTCATAAGACTGATCG
LvCTL4-F	CTTGGACGCTTATGTCACCTAC
LvCTL4-R	CATCCTTGCTCTTGATGTAGTCG
LvALF1-F	GGATGTGGTGTCCTGGATGG
LvALF1-R	GCGTCGTCCTCCGTGATG
LvALF3-F	GACCTGTCCAACCCTGAGC
LvALF3-R	TCGCCTCCTCCTCCGTTATC
LvALF4-F	CCTGGTGGCACTCTTCGC
LvALF4-R	ACGGTGAAGCGGCACTTATG
LvALF-AVK-F	GTTCCTGGTGGCACTCTTCG
LvALF-AVK-R	TCCGTCTCCTCGTTCCTCC
LvCrustin-F	CACAACCTGTTCCAACGACTAC
LvCrustin-R	ACCTGCGATCCGAAGAATGAG
cMnSOD-F	TTGCCGCTACGAAGAAGTTG
cMnSOD-R	AGAAGATGGTGTGGTTCAAGTG
PPO1-F	TCTTCGCCTCACGCATCTC
PPO1-R	TATCCTCACAGTCACCTCCTTC
PPO2-F	TCACGAACGCCGAGGAAC
PPO2-R	GCAGCCGCAGAAGTTGAAAC
PPOAE2-F	AGTCGTCCTTCATCCTTCCTTC
PPOAE2-R	GGATTGTGCGTTCCTGTTCAG
LvLys-F	CGGACTACGGCATCTTCCAG
LvLys-R	TCATCGGACATCAGATCGGAAC
LvLys-IT-F	GGTTGCGTCATGCCAGATG
LvLys-IT-R	GGTTTATGTGCGTCTTCCCAG
Vpa-16s	GGTGTAGCGGTGAAATGCGTAG
Vpa-16s	CCACAACCTCCAAGTAGACATCG
WSSV32678-F	GTTTTCTGTATGTAATGCGTGTAGG
WSSV32753-R	CCCACTCCATGGCCTTCA
TaqMan probe-WSSV32706	CAAGTACCCAGGCCCAGTGTCATACGTT
dsRNA production	
dsLvSWD4-F:	GAATAAGTGTGTGCGTGGTGTG
dsLvSWD4-R:	TCTGGCTCAACGCAAGATAAC
ds LvSWD4-T7-F:	GGATCCTAATACGACTCACTATAGG GAATAAGTGTGTGCGTGGTGTG
dsLvSWD4-T7-R:	GGATCCTAATACGACTCACTATAGG TCTGGCTCAACGCAAGATAAC
GFP-F	ATGGTGAGCAAGGGCGAGGAG
GFP-R	TTACTTGTACAGCTCGTCCATGCC
T7-GFP-F	GGATCCTAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGAG
T7-GFP-R	GGATCCTAATACGACTCACTATAGGTTACTTGTACAGCTCGTCCATGCC
dsLvSTAT-F	TCAGTATGCCCAGTCCTT
dsLvSTAT-R	CCTAACTCTTTCCGTCTCC
T7-dsLvSTAT-F	GGATCCTAATACGACTCACTATAGGTCAGTATGCCCAGTCCTT
T7-dsLvSTAT-R	GGATCCTAATACGACTCACTATAGGCCTAACTCTTTCCGTCTCC
dsLvIRF-F	CTTTCACCAATGTCCCGATG
dsLvIRF-R	CGGCGATGTCGTAGGAATG
T7-dsLvIRF-F	GGATCCTAATACGACTCACTATAGGCTTTCACCAATGTCCCGATG
T7-dsLvIRF-R	GGATCCTAATACGACTCACTATAGGCGGCGATGTCGTAGGAATG

Nucleotides in bold represent the restriction sites introduced for cloning.

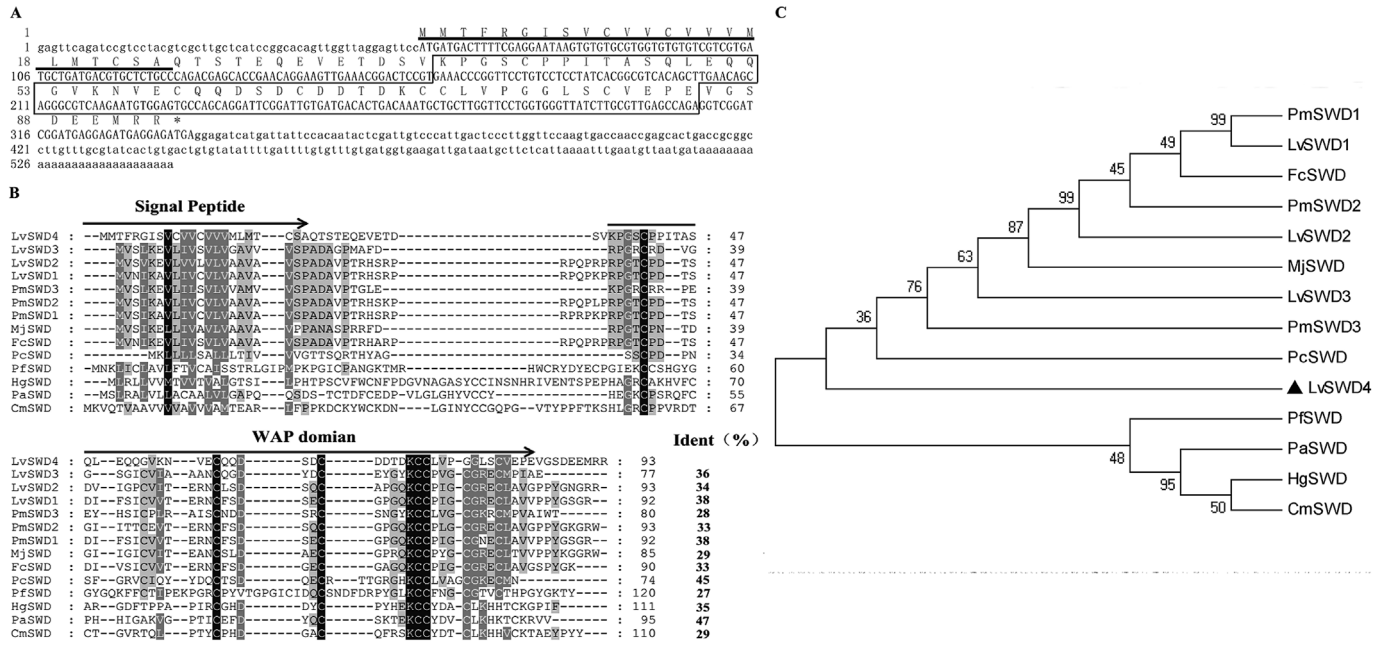

Fig. 1. Sequence analyses of LvSWD4. (A) Nucleotide and deduced amino acid sequences of LvSWD4 mRNA. The amino acid sequence was shown above the coding nucleotide sequence and numbered on the left. The signaling peptide and the WAP domain were underlined and boxed, respectively. (B) Multiple-sequence alignment of the deduced amino acid sequence of LvSWD4 with other crustacean SWDs. The identical amino acid residues were shaded in black and the similar residues in gray. (C) Neighbor joining phylogenetic tree analysis of the amino acid sequences of SWDs. LvSWD4 was marked with hollow triangle. Proteins analyzed included: PmSWD1, *Penaeus monodon* SWD protein isoform 1 (Genbank accession No. ACF28464); PmSWD2, *P. monodon* SWD protein isoform 2 (ACF28467); PmSWD3, *P. monodon* SWD protein isoform 3 (ACF28466); MjSWD, *Marsupenaeus japonicus* SWD protein (AU176270); FcSWD, *Fenneropenaeus chinensis* SWD protein (ABN09668), PcSWD, *Procambarus clarkii* SWD protein (ACY64753); PfSWD, *Pinctada fucata* WAP protein (AIU40990.1); LvSWD1, *Litopenaeus vannamei* SWD protein isoform 1 (AAS38513); LvSWD2, *L. vannamei* SWD protein isoform 2 (AAS17722); LvSWD3, *L. vannamei* single WAP domain-containing protein isoform 3 (AWD73559.1); HgSWD, *Homarus gammarus* putative Carcinin-like antibacterial protein (CAH10349.1); PaSWD, *Panulirus argus* PET15 (AAQ15293.1); CmSWD, *Carcinus maenas* carcinin (CAD20734.1).

species of the class Crustacea and is the major aquaculture shrimp in the world [15]. Because of its important evolutionary status and the sound research foundation on it, *L. vannamei* is now a useful model for studies on invertebrate immune system [16,17]. The JAK-STAT pathway has been characterized in *L. vannamei*, which has a critical implication in antiviral and antibacterial responses [18–20]. However, few target genes of the JAK-STAT pathway have been identified in *L. vannamei*, which may hinder the in-depth research on the immunity of shrimp. In the current study, a novel SWD gene (LvSWD4) was identified from *L. vannamei*, the expression of which was regulated by the JAK-STAT pathway. LvSWD4 was shown to have antiviral and antibacterial activities. This study could enrich the knowledge on the crustacean immunity and JAK-STAT pathway.

2. Materials and methods

2.1. Animals and pathogens

L. vannamei (~10 g) were from an aquaculture farm in Zhuhai, China and were randomly detected to ensure freeing of white spot syndrome virus (WSSV) and *Vibrio parahaemolyticus* by PCR as previously described [21,22]. Shrimp were acclimated at ~28 °C in a recirculating water tank system with air-pumped seawater (2.0% salinity) before experiments. The pathogens *V. parahaemolyticus* and WSSV were prepared as previously described [23].

2.2. Cloning of LvSWD4 gene

An expressed sequence tag (EST) with a WAP domain-encoding region was retrieved from a *L. vannamei* transcriptome library. The 3′ and 5′ end of LvSWD4 mRNA was amplified by rapid amplification of cDNA ends (RACE) using a SMARTer RACE cDNA Amplification kit (Clontech, Japan) and sequenced.

2.3. Bioinformatics analysis

The amino acid sequence of LvSWD4 was analyzed using BLAST software and its homologous genes were retrieved from the National Center for Biotechnology Information (NCBI) databases. Sequence alignments were made by Clustal W 1.8 with parameters as previously described [24]. Phylogenetic tree was generated by the neighbor-joining (NJ) method using MEGA 5.0 software with a Poisson model and other parameters as follows: substitution type (amino acid), rates among sites (uniform rates), gap missing deletion treatment (complete deletion), and number of bootstrap replication (1000 replicates). The promoter of LvSWD4 was analyzed using JASPAR software (http://jaspar.binf.ku.dk/).

2.4. Real-time PCR

To detect the distribution of LvSWD4 mRNA, tissues were sampled and pooled from 15 healthy *L. vannamei*. For challenge experiments, shrimp were intramuscularly injected with *Staphylococcus aureus* (10^6 CFU), *V. parahaemolyticus* (10^6 CFU), WSSV (10^6 copies), *Aspergillus niger* (10^5 CFU) in 50 μl PBS buffer. The PBS buffer containing no pathogens was also injected to generate a control group. The gills were sampled at 0, 4, 12, 24, 48, 72 and 96 h post injection. The RNA extraction, cDNA synthesis and real-time PCR were performed following the methods as previously described [25]. The EF-1α gene (Genbank accession No. GU136229) was used as internal control. Sequences of primers used in this study were listed in Table 1.

A

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   1 TGAAGCCAGT ACTCGGAAAA GAGTGGTAGC TCTGATCGGC AAACAAACCA CGCTGTAGCG TGTTTTTTGT TTGCAAGCAG CAGATTACGC GCAGAAAAAA
 101 AGGATTTCAA GAAGATCCTT TGATCTTTCT ACGGGGTCTG ACGCTCAGTG GAACGAAAAC TCACGTTAAG GGATTTTGGT CATGAGATTA TCAAAAAGGA
 201 TCTTCACCTA GATCCTTTTA TAGTCCGGAA ATACAGGAAC GCACGCTGGA TGGCCCTTCG CTGGGATGGT GAAACCATGA AAAAATGGCA GCTTCAGTGG
 301 ATTAAGTGGG GGTAATGTGG CCTGTACCCT CTGGTTGCAT AGGTATTCAT ACGGTTAAAA TTTATCAGGC GCGATTGCGG CAGTTTTTCG GGTGGTTTGT
 401 TGCCATTTTT ACCTGTCTGC TGCCGTGATC GCGCTGAACG CGTTTTAGCG GTGCGTACAA TTAAGGGATT ATGGTAAATC CACTTACTGT CTGCCCTCGT
 501 AGCCATCGAG ATAAACCGCA GTACTCCGGC CACGATGCGT CCGGCGTAGA GGATCGGGCG CGCCAACTGC TCTCTCTCAG CATCAAAGCG TGGATTCCCA
 601 TATTTTCGTT TTTTCGCGTA AATTCTTGTT TCTTATTGAA TGATAATTGT AAACAATCTC TTACGAGGTC ATTGATGAAT ATAAGTCACG TAAAGAAATC
 701 CAAACGATTA ATTATAGCCC ATCAATCAAT GGGCCTTCAA TTTCATTTGT ATTAAGATTT ACTCTTTTGA GACTAAAGAT AAGCATTATT CCATTCTGTA
 801 TAATCTTATA CCACTTTCAT AAGCATTTCA TAATAAATTA TCCTCTAGAT AATCCGTAAA TGTCCAAATA AGGAGTTACA AAATGAAATA GAAAATATAC
 901 CAGCATCAAT TATCTGGTAG GGTTTGTAAA CACTGAGAAG GTAAAACAGT GTGTGTCTTG AAGTTACATT TCTCTCCCTG TGCACTAGAC GCTGAAGTTA
1001 GTTTTCCGAA TAGAATGTGA CTTTTGACAT GCACATGACA TACTGGTTTA GAAGGTACGT GATTAAGCTA CAGGTTTCAG TCAACGCTCC TCATTAAAAG
1101 CAAAACAGAA AGGAGGTAAT GTGAGAGAGA TGAAATACAG CCATTGTGGA AAAAAATAAA ACCCGAGACT GTTTCGATAT GTTCCCCATT TATTTGCACA
1201 TCCTAATGAA GTAGAGAATA TATGCGGATT TGTGTGTGTG TTTGTGTATA TATATATATA TATATATATA TATATATATA TATATATATA
1301 TATATAAGAA AAAAAAAGGA AAAAATATAG TTTAGATACA AGGAAAGGTT TAGTTTCCAT TTGGGAAATC ACTTGCACGT GGGAGCCTCC GGGCACAATG
1401 CATGAATGAT AATAATTGCT TTGCTTCCTG ATGGTCATGG GAAAGGAAGG TTTAACCCTT CATATATCTC CAGATCGCGA AAAGAACCTG AATAATGGAA
1501 GCGATGGGTA GGCCTACAGG CCATGAAACA CATGAACGCT GATGGGAGTG CTTGGGCGCC TGGTGGAAAA TGTTTACACC TGTCCAGAAC ATTTTATTGA
1601 TCTACATATT GATATATTAC ACCTTTTCAA ATTTCAGAGA CAGATAATTA CTTTCAAACT CTTGTCTATC TACACTACCT ATCAAGGCCA AACAAGATCC
1701 GTCATTAGCT TTTGGCAATA GTCATATATT CTAAAAAGCA CAAAAGCAGT TCTAGTATTT TCATTTTATT CCTTTTTTGT AATAAATTGA TTTCTCATAA
1801 TTTATATTTA CAGGCAAACC ATCCCCGCAT CCACTTCACC CAGACTTTAT CCCAAGTGCC TTCAAGCACT CTACATTAAA TGAAAACAAA GCAGGTATAC
1901 AGTTGCCTGT TGCATTTGTG TCTGAAATGT CATGTGGTCC AGCAGAAGAA ATAACTGAAA AGGGCCTATC TCTTGAGGTC AAATTATTGA GGAGAGAGAA
2001 AAATGCGTTG TGCGCTTTTA GGCCTATACA TGACTTCATT GTCTCCTTTG TGTAAACACC TAGTATATGG GAAGAAAAGT GATATAAGGA AATGGCACTG
2101 ATTTCACAGA AAAATACTCT ATATAGCTAA ACGTCATACT TTACTGTAGC AAATTATGTT ATTACAAAAT TATTATCATC ATCATCTGCT TTATTGATAG
2201 AACAGTCGTA GGTGTCAGAA TATTCAATTT GAGGTCACTT CGAAGGATCG TCGATCCAGA TTTCGGTATG CGTACTCTTT TAGCTACACA GCTTCTAGTT
2301 TTGCTTCATA TCACCTTTTC GTTTCGGTTT TTGAGGAATC AAAGAATTCA TACACCGTTT TTCTTCACGT TGATAGCGAG GTATCTGTGA GCGACGGGTT
2401 TGTTTTCACC AAGTGGAATG GCGACTCCGA CTCGTGACAT CACACCGAAT CGGTCTATTC TCTTCAGAGC ATTATAGAAA ATATCTAGAA AAAGTAATGA
2501 TAATTTCCAA GAAATATATG CAAATGAATT TAGAGGTTTA GCATTTGCTA ATTCTATCCA TTGCCATAGC ATTTTTTTTC CTTTTTTTAT GGAAAAAGAT
2601 ATCCAAGTAA ACAAGCAGAA AACATAAATA TGTAAGAACA ATTTATGTAT CATCATTATT TTGGCGGGAG AAGCGATAAC AGTTTTGATA AGAAACGGTG
2701 TTTTTTTTTT TTTTAACGGC TTTATTCACA CACGTTCACC GGTCTCCCAA TTGCTTCTTC GCCTCGACAG AAAAAAAAGG TTTTGTCTTT ATCACTACGA
2801 GCTTCTATAA AACCATCTTT GCCTACCTAT AACATTTTGT ACGTGTGAAT AGCCACCTAT CTATGTTCTG AAATAAAATA TTCTGAAAAG TGCCACACAT
2901 TCAACATCAC AGTTTATCCT CTGCCTATCA ATCATTCAAA TGCATTCATA AATCTCTGTC CAACCCCAAA CAAATTCATT TTGTTTCCTA ACACGGTTTC
3001 GCAACACCGA CCATTTTCGC CATAACACCG GCTCTTCGGT GGATAAAAGG GCAGCGCTTG GCCCCTTGAT GCTTCAGTTC AGATCCCGGA AG
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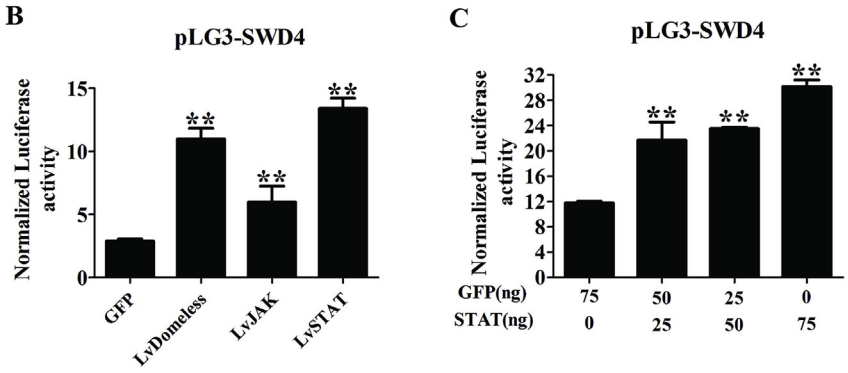

Fig. 2. Regulation of the LvSWD4 promoter by the JAK-STAT pathway. (A) Sequence analysis of the LvSWD4 promoter. The predicted TATA-box was boxed and the putative STAT binding sites were underlined. (B) The regulatory effects of STAT, Domeless and JAK on the LvSWD4 promoter detected by dual-luciferase reporter assays. (C) Effects of gradient transfection of STAT-expressing plasmids on the LvSWD4 promoter detected by dual-luciferase reporter assays. Each bar represents the mean ± SD (n = 8) (*: $p < 0.05$, **: $p < 0.01$).

2.5. Dual luciferase reporter assays

The promoter of LvSWD4 was cloned using a genome walking strategy and cloned into the firefly luciferase plasmid pGL3-Basic (Promega, USA) as previously described [26]. The ORFs of *L. vannamei* Domeless, JAK and STAT genes were cloned into pAc5.1/V5-His A plasmid (Invitrogen, USA) to generate their expression vectors. For the dual luciferase reporter assays, S2 cells were plated in 96-well plate and transfected using 0.05 μg reporter Firefly luciferase plasmid, 0.1 μg gene expression vector, and 0.03 μg pRL-TK renilla luciferase plasmid (as internal control) (Promega, USA). 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). Three independent experiments were performed and all assays were performed in eight independent transfections.

2.6. In vitro antibacterial assays

The coding sequence of LvSWD4 without the signal peptide was cloned into the pET-32a (+) vector (Merck Millipore, Germany) and transformed into the *Escherichia coli* strain BL21 (DE3) (Invitrogen, USA) and induced using IPTG. The recombinant 6His-tagged LvSWD4 protein was purified with Ni-NTA agarose (Qiagen, Germany) under native conditions and dialyzed against PBS buffer. The recombinant Trx protein expressed by the empty pET-32a (+) vector was purified as control. The antibacterial activity of LvSWD4 against *V. parahaemolyticus, E. coli*, *Staphylococcus aureus*, and *Bacillus subtilis* was examined following a previously described method [27]. Briefly, Bacteria were cultured in LB medium to a midlogarithmic phase. The bacterial suspension was diluted to 5×10^4/ml with LB medium containing gradient concentrations of LvSWD4 or Trx protein. The 0.1 μg ampicillin and PBS buffer (10 μl) were used as positive and negative controls, respectively. After incubation at 37 °C for 16 h, the bacterial concentration was determined by absorbance at 600 nm (A600).

2.7. RNA interference

Double stranded RNAs (dsRNAs) specific to LvSWD4 (dsRNA-LvSWD4) and green fluorescent protein (dsRNA-GFP, as control) were prepared by *in vitro* transcription using a T7 RiboMAX™ Express RNAi System (Promega, USA) as previously described [28]. Shrimp were injected with 50 μl PBS containing 5 μg dsRNA-LvSWD4 or dsRNA-GFP (as control). At 48 h post injection, the RNA interference (RNAi) efficiency of LvSWD4 and the expression of many immune related genes in gills were detected using real-time PCR with primers listed in Table 1. For immune challenge experiments, at 48 h post dsRNA injection,

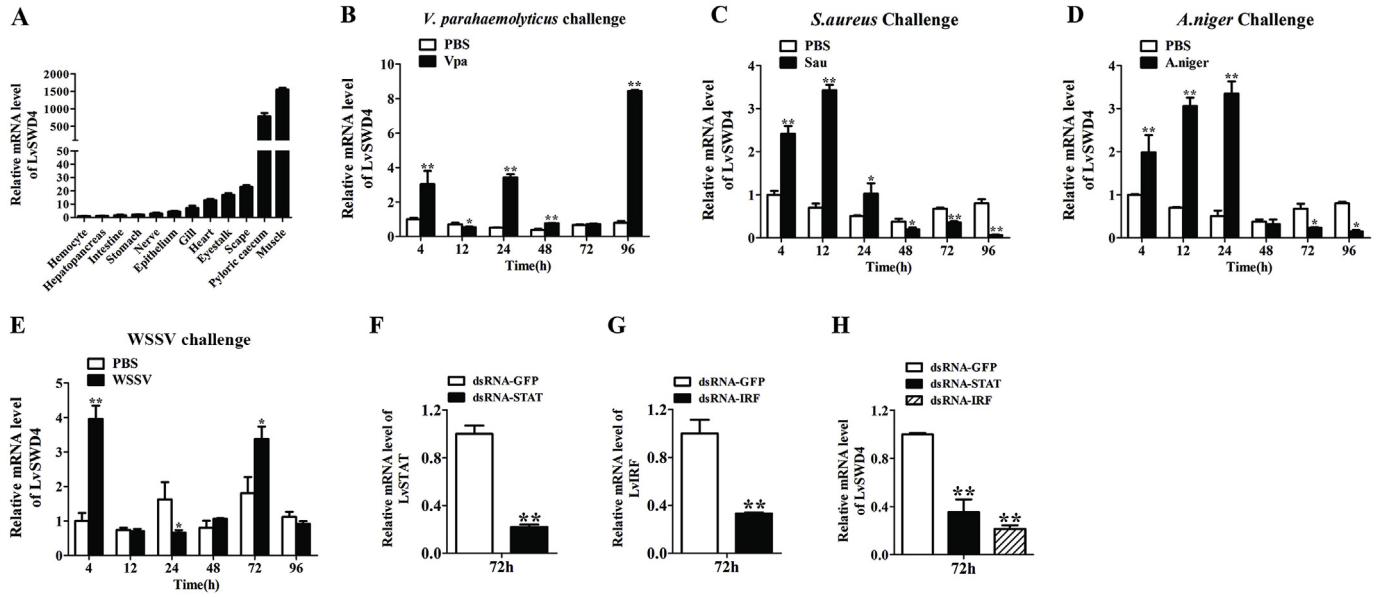

Fig. 3. Expression profiles of LvSWD4 analyzed using real-time PCR. Each bar represents the mean ± SD of the three detections. (A) The transcription of LvSWD4 *L. vannamei* tissues. The mRNA levels were calculated relative to that in hemocytes (set as 1.0). (B–E) The expression profiles of LvSWD4 in gills of *V. parahaemolyticus*-, *S. aureus*-, *A. niger*-, and WSSV-challenged shrimps were examined. (F and G) The knock-down efficiencies of STAT and IRF. (H) The mRNA level of LvSWD4 in STAT- and IRF-silenced shrimp. The statistical significance was calculated using Student's *t*-test. *, $p < 0.05$, **, $p < 0.01$.

shrimp were further injected with 10^6 copies of *V. parahaemolyticus* and WSSV ($n = 50$ in each group). Experiments were done in triplicate and the cumulative mortality was recorded. At 6 h and 12 h post *V. parahaemolyticus* infection, 96 h and 120 h post WSSV infection, the gill and muscle tissues were sampled and the copy number of *V. parahaemolyticus* and WSSV were determined using quantitative real-time PCR as previously described, respectively [28].

2.8. Phagocytic activity analysis

The phagocytic activity of hemocytes from shrimp at 48 h post dsRNA injection was analyzed following the method as previously described [25]. Briefly, hemocytes extracted from dsRNA-treated shrimp were washed with 2 × Leibovitz's L-15 medium (Gibco, USA) triply and mixed with fluorescein isothiocyanate (FITC)-labled *V. parahaemolyticus* at a 1:100 ratio of cells/bacteria and incubated at 28 °C for 1 h. Hemocytes was then detected using cytometry for the signals of FITC and the forward scatter (FSC) values of cells. The FSC threshold was determined by detecting the free FITC-labeled *V. parahaemolyticus* to eliminate cell debris and bacteria, and the fluorescence boundary was set based on detection of the self-fluorescence of untreated hemocytes. A total of 500,000 events were detected for each sample.

2.9. Statistical analysis

The statistical procedures were carried out using SPSS statistical software version 16.0. The mean and standard deviation (SD) from three detections was calculated. Student's t-test was used to compare the two means. The Kaplan-Meier plot (log-rank $\chi 2$ test) was used to analyze the mortalities between different groups.

3. Results

3.1. Cloning and bioinformatics analysis of LvSWD4

The ORF of LvSWD4 gene is 282 bp encoding a protein of 93 amino acids (GenBank Accession No. MK354273). The LvSWD4 protein was predicted to possess an N-terminal signaling peptide (residues 1–23) and a single WAP domain in the C-terminal region (Fig. 1A). The predicted mature LvSWD4 protein without the signal peptide has a calculated molecular weight of 7.42 kDa and a theoretical isoelectric point (pI) of 3.95. Multiple-sequence alignment indicated that the LvSWD4 protein shared 38%, 34% and 36% identities with the three previously reported *L. vannamei* SWDs LvSWD1, LvSWD2 and LvSWD3, and 38%, 33% and 28% with PmSWD1, PmSWD2 and PmSWD3 from black tiger shrimp *Penaeus monodon*, respectively. Among the analyzed sequences, the highest homology (47%) was observed between LvSWD4 and the *Panulirus argus* PaSWD. LvSWD4 also showed 27% homology with a SWD from pear oyster *Pinctada fucata* (Fig. 1B). Phylogenetic tree analysis showed that the distance between LvSWD4 and other shrimp SWDs was relative large (Fig. 1C). Considering its low pI which is different with other SWDs, these suggested that LvSWD4 could be a relatively unique member of the shrimp SWD family.

3.2. Promoter analysis of LvSWD4

The upstream 3,092-bp sequence upstream the transcriptional initiation site of LvSWD4 amplified using genome walking method was predicted to contain five potential STAT-binding motifs, indicating that the expression of LvSWD4 could be regulated by STAT (Fig. 2A). This sequence was then cloned into the PGL3 vector and subjected to dual-luciferase reporter assays. Compared with the control, expression of STAT and the two important components of the JAK-STAT pathway Domeless and JAK could significantly up-regulated the activity of LvSWD4 promoter (Fig. 2B). With the increase of the levels of transfected STAT-expressing plasmids, the activity of LvSWD4 promoter was also up-regulated (Fig. 2C). The dose-independent manner in which STAT regulated the LvSWD4 promoter confirmed that LvSWD4 was a regulatory target gene of STAT.

3.3. Tissue distribution and expression profile of LvSWD4

The tissue distribution of LvSWD4 mRNA in healthy shrimp was analyzed using real-time PCR (Fig. 3A). The lowest level of LvSWD4 mRNA was detected in hemocytes and the highest levels were detected in muscle and pyloric caecus, which were up to 1548.5- and 786.9-fold higher than that in hemocytes, respectively. In contrast, other tissues expressed 1.3- to 23.0-fold levels of LvSWD4. The expression of

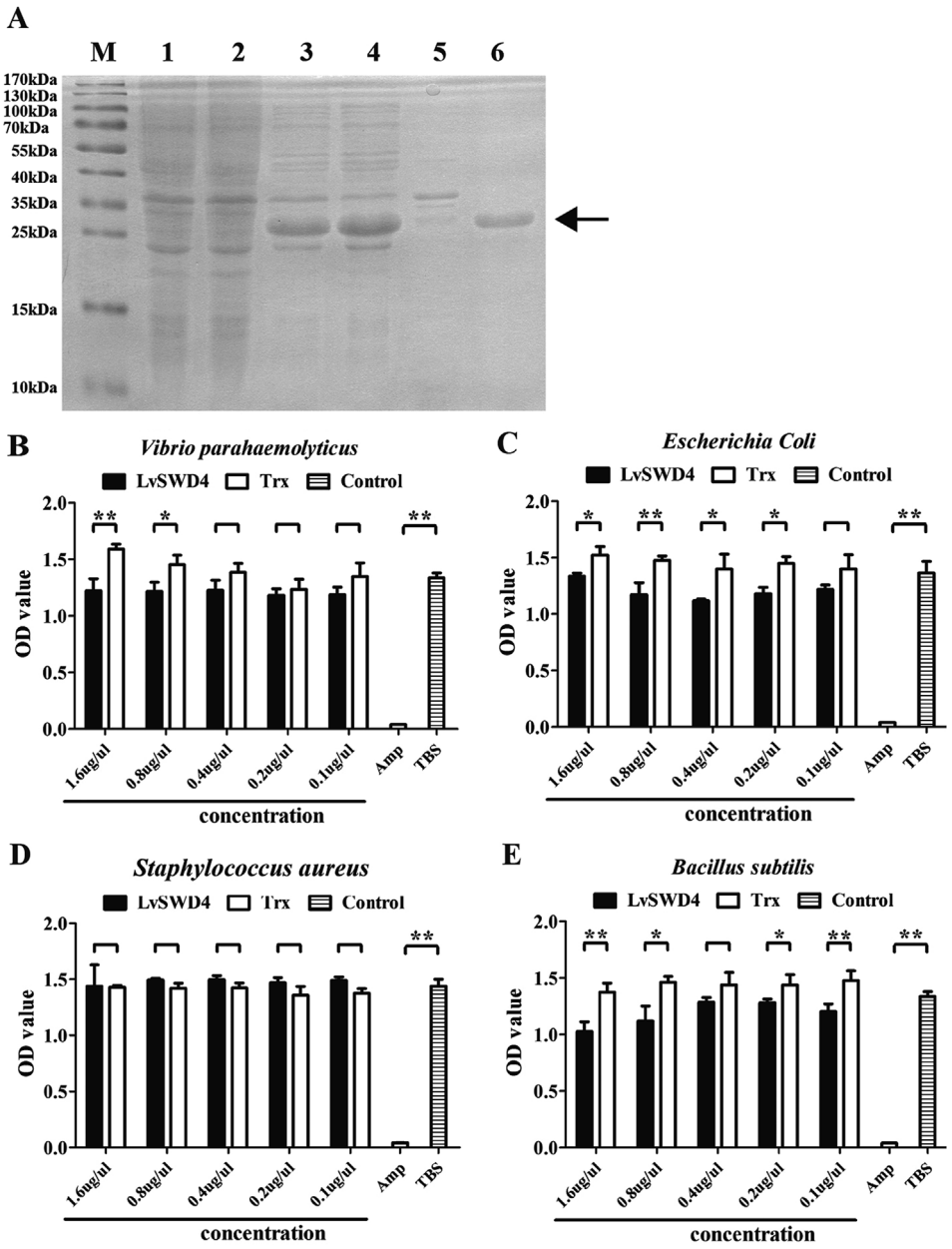

Fig. 4. *In vitro* antibacterial assays of LvSWD4. (A) SDS-PAGE analysis of the recombinant LvSWD4 protein expressed in *E. coli*. Line 1, untransformed *E. coli* cells; Line 2, uninduced *E. coli* transformed with LvSWD4; Line 3, induced *E. coli* transformed with LvSWD4; Line 4, supernatant of ultrasonic lysed *E. coli* expressing LvSWD4; Line 5, precipitate of lysed *E. coli* expressing LvSWD4; Line 6: purified recombinant LvSWD4 protein (black arrow). (B–E) Effects of gradient concentrations of recombinant LvSWD4 and Trx (negative control) proteins and 0.1 μg ampicillin (Amp, positive control) on the growths of *V. parahaemolyticus, E. coli*, *S. aureus*, and *Bacillus subtilis*. The bacterial concentration was determined by absorbance at 600 nm (A600). Each bar represents the mean ± SD of three samples. *, $p < 0.05$, **, $p < 0.01$.

LvSWD4 in gill, an important immune tissue of shrimp, during immune stimulation was further investigated (Fig. 3B–E). The increased expression of LvSWD4 was mainly observed at 4, 24 and 96 hpi in *V. parahaemolyticus*-challenged shrimp and at 4 and 72 hpi in WSSV-challenged shrimp, while in *S. aureus*- and *A. niger*-infected shrimp, the increase of LvSWD4 expression mainly occurred at 4–24 hpi. The regulatory effect of STAT on LvSWD4 expression was further explored *in vivo*. The expression of STAT and IRF was silenced using RNAi strategy and the mRNA level of LvSWD4 was analyzed (Fig. 3F). Compared with the control, silencing of STAT significantly decreased the expression of LvSWD4 (Fig. 3H). As the transcription factor IRF has been known as an upstream regulator of the JAK-STAT pathway in shrimp [17], we further knockdown the expression of IRF and analyzed the mRNA level of LvSWD4 *in vivo* (Fig. 3G). The result demonstrated that the expression of LvSWD4 in IRF-silenced shrimp was significantly down-regulated (Fig. 3H).

3.4. Immune function of LvSWD4

The LvSWD4 protein was recombinantly expressed in *E. coli* and purified using affinity chromatography (Fig. 4A). The antibacterial activity of recombinant LvSWD4 protein was investigated *in vitro* (Fig. 4B–E). The result showed that compared with the control Trx protein, LvSWD4 significantly inhibited the growth of Gram negative bacteria *V. parahaemolyticus* and *E. coli* and Gram positive bacterium *B. subtilis*. In contrast, no significant difference was observed between growths of LvSWD4- and Trx-treated *S. aureus*. To investigate the immune role of LvSWD4 *in vivo*, RNAi strategy was used to knockdown the expression of LvSWD4 in shrimp (Fig. 5A). Compared with the control, silencing of LvSWD4 significantly increased the mortality of shrimp infected with WSSV and *V. parahaemolyticus* (Fig. 5B and D). Consistent with these, the copy numbers of WSSV in muscle and *V. parahaemolyticus* in gill were also significantly increased after silencing of LvSWD4 (Fig. 5C and E). These suggested that LvSWD4 could play positively roles in both antiviral and antibacterial immune responses.

To further explore the role of LvSWD4 in shrimp immunity, the

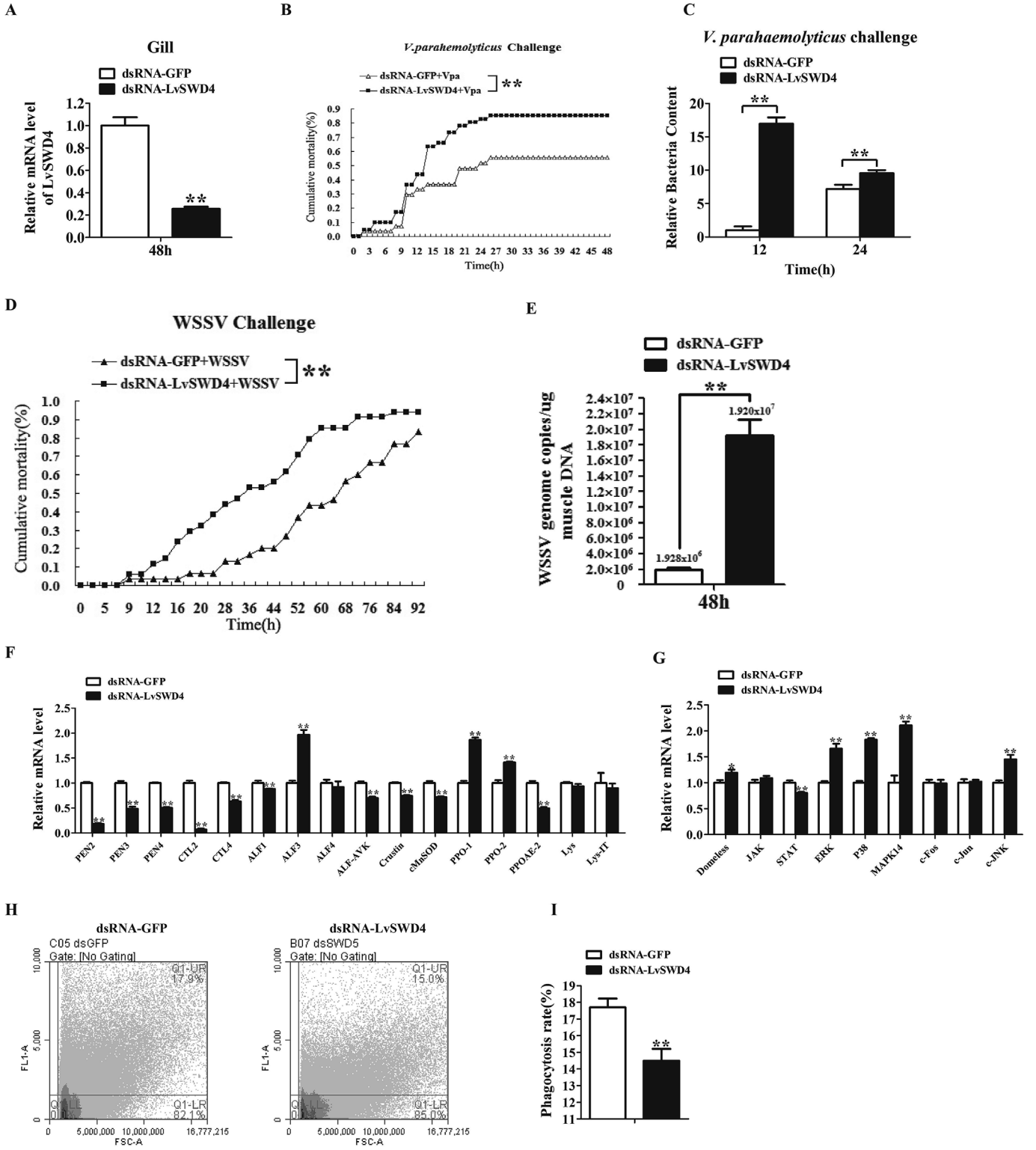

Fig. 5. Involvement of LvSWD4 in immune responses. (A) The knock-down efficiency of LvSWD4 in gill at 48 h post dsRNA injection. (B and D) Mortalities of LvSWD4-dsRNA and control GFP-dsRNA treated *L. vannamei* (n = 50) infected by *V. parahemolyticus* and WSSV. Cumulative mortalities were recorded every 4 h. Differences in cumulative mortality levels between treatments were analyzed by Kaplan-Meier log-rank χ^2 tests (**$p < 0.01$). (C and E) The relative bacterial load in gill and the absolute viral load in muscle detected by quantitative real-time PCR. Each bar represents the mean ± SD of three samples (**$p < 0.01$). (F), expression of various immune related genes in LvSWD4-silencing shrimp analyzed by real-time PCR. Each bar represents the mean ± SD of three samples. (G and H) Flow cytometry analysis of the phagocytic activities against FITC-labeled *V. parahaemolyticus* of hemocytes from SWD-4 silenced shrimp. The scatter plots representing one of the three flow cytometric detections were shown in the left panel and the data were provided in the right panel. Cells were examined by forward scatter (FSC, x-axis) and the phagocytosis of FITC-labeled *V. alginolyticus* was indicated by intracellular green fluorescence (y-axis). **$p < 0.01$.

expression of various immune functional genes in LvSWD4-silenced shrimp was analyzed using real-time PCR (Fig. 5F). The results demonstrated that after silencing of LvSWD4, expression of most of the detected AMPs, such as PEN2, PEN3, PEN4, ALF1, ALF-AVK, and Crustin was significantly down-regulated, but expression of ALF3 was significantly up-regulated and expression of ALF4 did not show significant change. Meanwhile, expression of some immune functional proteins including C-type lectin 2 (CTL2), CTL4, cytosolic manganese superoxide dismutase (C-MnSOD), and polyphenoloxidase PPOAE-2 was also significantly decreased, but expression of polyphenoloxidases PPO-1 and PPO-2 was significantly increased. In contrast, there was no significant change in the expression of lysozyme (Lys) and invertebrate lysozyme (Lys-IT). The influence of LvSWD4 on the phagocytic function of shrimp hemocytes was further investigated (Fig. 5G and H). The results showed that the phagocytic activity of hemocytes from LvSWD4-silenced shrimp against *V. parahaemolyticus* was significantly lower than those from the control shrimp, suggesting that LvSWD4 could positively regulate the phagocytic activity of hemocytes. Taking together, these results suggested that LvSWD4 could be implicated in regulation of both humoral and cellular immune responses.

4. Discussion

There are four JAK proteins (JAK1, JAK2, JAK3 and TYK2) and seven STAT proteins (STAT1-4, STAT5a and 5b, and STAT6) in human, indicating a complex mechanism underlying the signal transduction of the JAK-STAT pathway [29,30]. Similarly to that in *Drosophila*, the JAK-STAT pathway in *L. vannamei* has fewer components but can sufficiently mediate signal transduction analogous to that of vertebrates. There are only one STAT, one JAK and two negative regulators SOSCs identified so far in the JAK-STAT pathway in *L. vannamei* [19,20,31]. Besides, a cytokine receptor (Domeless) upstream of the JAK protein was also identified in *L. vannamei*, which played an important role in antiviral responses [18]. The promoter evidenced to be regulated by STAT is the promoter of the *ie1* gene (wsv069) from the shrimp viral pathogen WSSV, which has been widely used in studies on the JAK-STAT pathway in shrimp. However, few STAT-targeted genes from shrimp have been reported up to now [32]. In the current study, the promoter of the novel identified LvSWD4 gene was predicted to have multiple STAT-binding sites and was confirmed to be regulated by STAT and the JAK-STAT pathway components JAK and Domeless by dual luciferase reporter assays. The RNAi knockdown experiment also demonstrated that silencing of STAT and the JAK-STAT pathway upstream regulator IRF led to significant decrease of the LvSWD4 expression. These suggested that LvSWD4 was a novel regulatory target gene of the JAK-STAT pathway in shrimp.

SWDs are a group of single WAP domain-containing crustins found in invertebrates. At present, a growing number of attempts have been made to characterize the SWDs in shrimp, including those from *Litopenaeus vannamei, Penaeus monodon, Fenneropenaeus chinensis* and *M. japonicus* [33–37]. These proteins shared similar structure and high sequence homology. Studies in *P. monodon* and *F. chinensis* suggested that SWDs could inhibit the infection of Gram-positive and -negative bacteria through attenuating the hydrolysis activity of proteinases derived from bacteria [34,38]. The expression of these shrimp SWDs could be stimulated by WSSV infection but their roles in antiviral responses have not been investigated. Most of the identified crustacean SWDs have isoelectric points above neutral pH. A recent identified SWD protein from *L. vannamei* (LvSWD3) with a low isoelectric point did not show proteinase inhibitory and antibacterial activities but had a verified antiviral activity [39]. In the current study, *in vitro* experiments demonstrated that the recombinant LvSWD4 protein had significant inhibitory activities against Gram negative bacteria *V. parahaemolyticus* and *E. coli* and Gram positive bacterium *B. subtilis*, suggesting a broad-spectrum antibacterial activity of LvSWD4. Knockdown of LvSWD4 *in vivo* resulted in high susceptibility of shrimp to the infection by *V. parahaemolyticus*. Also, LvSWD4 showed a significant antiviral activity against WSSV infection *in vivo*. Therefore, LvSWD4 could be the first member of the type III crustin group that has been showed to have both antibacterial and antiviral activities. Moreover, previous studies mainly focused on the role of the JAK-STAT pathway in antiviral responses. A recent study in *M. japonicus* showed that knockdown of STAT by RNAi declined the survival rate of *V. anguillarum*-infected shrimp and resulted in impaired bacterial clearance, suggesting an important role of the JAK-STAT pathway in antibacterial responses in shrimp [40]. Therefore, targeting LvSWD4 could be a mechanism underlying the antibacterial and antiviral functions of the JAK-STAT pathway, which requires further investigation.

It has been known that AMPs can function as immunomodulators to regulate innate immune responses through enhancing production of proinflammatory mediators, chemoattracting inflammatory and regulating complement activation in mammals [41–43]. In adaptive immunity, AMPs can recruit antigen-presenting dendritic cells, naïve T-cells, and memory T-cells to infected regions and can act on mast cells and macrophages to stimulate repair and clean-up of infected sites [44–46]. However, few studies concern the immune regulatory activity of AMPs in invertebrates. The current study demonstrated that knockdown of LvSWD4 *in vivo* could attenuate the phagocytosis of hemocytes, suggesting that LvSWD4 could positively regulate the cellular immune responses in shrimp. Furthermore, expression of many AMPs and immune functional genes was significantly down-regulated in LvSWD4-silenced shrimp, indicating that LvSWD4 could regulate the expression of these genes. Taking together, the evidence provided in this study demonstrated that a shrimp AMP LvSWD4 could play important roles in regulation of both humoral and cellular immune responses, which could be part of the mechanism underlying its antiviral and antibacterial functions.

Acknowledgements

This work was funded by the National Key Research and Development Program of China (2018YFD0900505); National Natural Science Foundation of China under grant No. 31572649, 31772881 and 31702371; Natural Science Foundation of Guangdong Province, China 2014A030313125; China Agricultural Research System CARS47; The Key Fundamental Research Funds for the Central Universities of China 17lgzd27.

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