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JAK/STAT signalling regulates antimicrobial activities in *Eriocheir sinensis*

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ABSTRACT

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) signalling pathway plays a significant role in immune responses to pathogens. In invertebrates, three core components (Domeless, Hopscotch and STAT92E) of the JAK/STAT pathway were first identified in *Drosophila melanogaster*. In the present study, we report the cloning and characterisation of DOME, JAK and STAT from *Eriocheir sinensis*. *Es*DOME, *Es*JAK and *Es*STAT are 1299, 1110 and 794 amino acid proteins encoded by 4200, 3333 and 2385 bp open reading frames, respectively. Bioinformatics analysis revealed that all three share specific domains with corresponding functions. Quantitative real-time PCR showed that all three components were highly expressed in various tissues including the gill, hepatopancreas and hemocytes. Moreover, *Es*DOME, *Es*JAK and *Es*STAT were upregulated significantly in hemocytes after bacterial challenge. In addition, the JAK/STAT signalling pathway positively regulates antimicrobial peptide expression during the antibacterial immune response. Thus, the JAK/STAT pathway plays a critical role in bacterial immune responses in *E. sinensis*.

1. Introduction

Innate immunity plays a very important role in combating microbial infection in all animals, especially invertebrates [1,2]. Drosophila mounts a potent host defence when challenged by various microorganisms, and the Toll and IMD signalling pathways are pivotal in response to Gram-positive and Gram-negative bacteria, respectively. These pathways participate in various immune responses, including controlling the expression genes encoding antimicrobial peptides (AMPs) that can kill numerous types of microorganisms [3–5]. Different Toll-like receptors recognise signals from different Gram-positive bacteria, such as Lys-type peptidoglycan (PGN), or fungi, such as β-1,3-glucans. In the IMD pathway, recognition of Gram-negative bacterial infection in Drosophila is mediated, in part, by the putative transmembrane receptor PGRP-LC, which activates NF-kB (Relish) that in turn regulates expression of AMPs after entering the nucleus through a signal transduction pathway [6]. In addition to Toll and IMD signalling pathways, the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway is of great significance in cellular immune responses [7]. However, relatively few related studies on this pathway have been reported in invertebrates.

The JAK/STAT pathway plays a significant role in cell proliferation, differentiation, apoptosis, and immune regulation. It is mainly composed of three important components; tyrosine kinase-related receptors, the tyrosine kinase JAK, and STAT [7]. In mammals, receptors located on the cell membrane are activated by ligands such as cytokines and growth factors, including interleukins 2–7 (IL-2 − 7), granulocyte-macrophage colony stimulating factor (GM-CSF), growth hormone (GH), epidermal growth factor (EGF) and interferon (IFN).

After ligand binding, receptor complexes phosphorylate their corresponding JAK tyrosine kinase. The JAK protein family consists of JAK1, JAK2, JAK3 and Tyk2, all of which possess seven JAK homology (JH) domains. The JH1 domain is a kinase domain, while JH6 and JH7 are receptor binding domains. The JAK tyrosine kinase recruits and phosphorylates STAT family proteins STAT1 − 4, STAT5a, STAT5b and STAT6. Activated STATs enter the nucleus as a dimer and bind to their target genes, thereby regulating transcription. Phosphorylated STATs dimerise and translocate from the cytoplasm into the nucleus to activate the expression of specific genes [8].

In Drosophila, the pivotal components of the JAK/STAT pathway are simpler than in mammals, and include three unpaired ligands (Upd1, Upd2 and Upd3), the cytokine-like transmembrane receptor Domeless (Dome), the JAK kinase Hopscotch (HOP) and a STAT named STAT92E [7]. Several negative regulators of JAK/STAT pathway are also found, for example, Latran is a dominant-negative receptor that antagonizes Dome function; Suppressor of cytokine signalling at 36E (Socs36E) inhibits Hop activity; PTP61 probably dephosphorylates STAT92E; Protein inhibitors of activated stats (PIAS) binds to and

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inhibits STAT92E activity; Ken and barbie (KEN) competes with Stat92E at the promoters of a subset of JAK/STAT target genes and represses them via nucleosome remodeling factor (NURF) recruitment; NURF regulates the transcriptions of the JAK/STAT components epigenetically [9]. In Drosophila, the JAK kinase HOP is involved in the control of viral load, and particular viral infections induce STAT DNA-binding activity [7]. Activation of the JAK/STAT pathway in response to bacterial infection has also been reported in insects [10,11]. For example, STATs are activated in response to bacterial infection in Anopheles [11]. After stimulation with lipopolysaccharide (LPS), STATs stimulate tyrosine phosphorylation and DNA-binding activity in *Aedes albopictus* and *Culex tritaeniorhynchus* mosquitoes, and increase luciferase activity of a reporter gene containing the Drosophila STAT binding motif in mosquito C6/36 cells [10]. Furthermore, the JAK/STAT pathway participates in antiviral and antibacterial immune responses in crustaceans.

In Pacific white shrimp (*Litopenaeus vannamei*), JAK/STAT pathway is widely reported to regulate innate immune responses [12–18]. For example, *Lv*Dome activates the JAK/STAT pathway [15], and *Lv*JAK plays an important role in the defence against white spot syndrome virus (WSSV) [13]. Besides, LvGRIM-19 appears to participate in innate immune responses as it interacts with the transcription factor STAT and effects JAK/STAT pathway [18]. Moreover, several other regulators of JAK/STAT pathway in Pacific white shrimp are also found, such as *Lv*SOCS2 [14], TC-PTP (TC45) interacted withβ-Arrestin 1 [16]. In the kuruma shrimp (*Marsupenaeus japonicus*), a structurally novel C-type lectins (*Mj*CC-CL) as a ligand of the receptor Dome directly activates the JAK/STAT pathway in the shrimp immune response to bacterial infection [17]. In addition, a lectin directly activates the JAK/STAT pathway to upregulate expression of selected AMPs during shrimp antibacterial immune responses by cross-linking microbial pathogens with the Dome cell surface receptor [16,17].

Chinese mitten crab (*Eriocheir sinensis*) is one of the most economically important aquatic animals in southeast Asia [19], but aquaculture of this species is threatened by a large number of diseases, resulting in low yields and severe economic losses [20]. There are many reports on the innate immunity of *Eriocheir sinensis* [21–30], and diseases caused by bacteria are the most damaging, and studies of innate immune defences in crustaceans revealed that AMPs are essential factors in the clearance of immune responses from pathogenic microorganisms [31,32]. In the present study, we obtained cDNAs from three important components in the JAK/STAT signalling pathway of *E. sinensis*, namely *Es*DOME, *Es*JAK and *Es*STAT. We also conducted bioinformatics analysis of these three genes, and measured mRNA and protein expression levels. Additionally, we investigated the expression patterns of JAK/STAT pathway components, which are dominated by these three genes, following pathogen attack, and the antibacterial functions related to regulation of AMPs in innate immune defences. RNA interference of *Es*DOME and inhibition of the *Es*STAT protein downregulated AMP gene expression. Therefore, the JAK/STAT pathway is closely related to antibacterial immune responses in *E. sinensis*, and is involved in regulating the expression of AMPs.

2. Material and methods

2.1. Animals

Healthy adult Chinese mitten crabs (n = 240; wet weight = 100 ± 15 g) were purchased form a local agricultural market in Shanghai, China, and temporarily farmed in fresh water at a suitable constant temperature (20–25 °C) before use in experiments.

2.2. Bacterial challenge and sample collection

The Gram-negative bacterium *Staphylococcus aureus* (BYK0113) and the Gram-positive bacterium *Vibrio parahaemolyticus* (BYK00036) were obtained from the National Pathogen Collection Center for Aquatic Animals (Shanghai Ocean University, Shanghai, China). Bacteria were cultured for 12 h in Luria-Bertani (LB) medium and collected by centrifugation (5000 × g for 3 min), washed three times in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), resuspended in PBS, and plated for colony counting. For immune challenge experiments, 240 crabs were randomly divided into three groups (sex ratio = 1:1) and injected with *S. aureus* (100 mL), *V. parahemolyticus* (100 mL) or PBS (100 mL). After diluting bacteria to 1×10^6 colony-forming units (CFU)/mL, the bacterial suspension (100 mL) was injected into each crab in the *S. aureus* and *V. parahaemolyticus* challenge groups, while crabs in the control group were injected with 100 mL PBS. At specific time points (0, 2, 4, 8, 12 h, 24 and 36 h) after bacterial challenge, we randomly selected more than five crabs from each group and collected hemolymph from hemocoel in the arthrodial membrane of the last two pairs of walking legs of each crab using a sterile syringe with 5 mL of anticoagulant solution (0.1 M glucose, 30 mM citrate, 26 mM citric acid, 0.14 M NaCl, and 10 mM ethylenediaminetetraacetic acid) [33]. Hemocytes were then obtained by centrifugation (800 × g, 4 °C, 10 min) and the collected hemolymph samples were stored at −80 °C. Heart, muscle, stomach, intestine, gill, hepatopancreas and hemocyte samples were collected from the control group, immediately frozen in liquid nitrogen and stored at −80 °C. According to a previous study in our laboratory [34], *E. sinensis* primary hemocytes were cultured and collected.

2.3. Total RNA extraction and gene cloning of EsDOME, EsJAK and EsSTAT

Total RNA was extracted from *E. sinensis* using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and our previous publication [35]. We used a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and 1% agarose gel electrophoresis (AGE) to examine the concentration and quality of total RNA.

For gene cloning, total RNA (5 μg) was reverse-transcribed using a SMARTer rapid amplification of cDNA ends (RACE) cDNA Amplification kit (Clontech, Shiga, Japan). We obtained partial cDNA sequences in the form of expressed sequence tags (ESTs) for *Es*DOME, *Es*JAK and *Es*STAT from the hemocyte cDNA library of the Chinese mitten crab (unpublished). Partial *Es*DOME, *Es*JAK and *Es*STAT cDNA sequences were extended by 5′ and 3’ RACE using the SMARTer RACE cDNA Amplification kit (Clontech, Shiga, Japan) according to the instructions of the manufacturer, and gene-specific primers (Table 1) were designed based on the *E. sinensis* original partial sequences and synthesised by RuiMian (Shanghai, China). Amplification was performed at 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 32 s, and 72 °C for 2 min, and a final extension at 72 °C for 12 min. PCR and RACE-PCR were purified and inserted into the pEASY-T1 vector (TransGen, China) for DNA sequencing. Nucleotide sequences of *Es*DOME, *Es*JAK and *Es*STAT have been submitted to GenBank under accession numbers MH294434, MH294435 and KF471408, respectively.

2.4. Bioinformatic analysis

The obtained sequences were compared against other sequences from invertebrates and vertebrates in the NCBI database using the online search tool BLASTX (http://www.ncbi.nlm.nih.gov/) and open reading frames (ORFs) were identified using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). SMART (http://smart.embl-heidelberg.de/) was used to predict the structure and function of domains, and SWISS-MODEL (http://swissmodel.expasy.org/) was used to construct three-dimensional models. Multiple sequence alignment was performed using the ClustalX 2.0 program and DNAMAN software. Phylogenetic trees of *Es*DOME, *Es*JAK, *Es*STAT and other related

Table 1
Sequences of primers used for EsDOME, EsJAK and EsSTAT analysis.

Primer name and purpose	Primer sequence (5′-3′)
cDNA cloning	
*Es*DOME-3RACE1	CAGCCCTATGTTGAGTATGAGTT
*Es*DOME-3RACE2	CCTTGTGACGACCAAACCCAGCAT
*Es*DOME-5RACE1	CTGGGTTTGGTCGTCACAAGGAT
*Es*DOME-5RACE2	CTGCCTCTTCATCGCATTCATAGGG
*Es*JAK-3RACE1	CTCACCTATGCGGACGGTTCTGT
*Es*JAK-3RACE2	GGGTCCCGTGCTACTTCAAACTG
*Es*JAK-5RACE1	AATCCATTCCCTCAGCGATGTCTAT
*Es*JAK-5RACE2	GTGGCACGGAGGCTTCAGGTTCA
*Es*STAT-3RACE1	TCTTGGTGCGGATACGCCTTGAT
*Es*STAT-3RACE2	TGCCGACCCTCAACTCCCACATC
*Es*STAT-5RACE1	TCACCCAGTGGACGGATGGCAAA
*Es*STAT-5RACE2	GGTAACAAGCGAGGACAGGAGGC
Quantitative real-time PCR	
β-actin qF	GCATCCACGAGACCACTTACA
β-actin qR	CTCCTGCTTGCTGATCCACATC
*Es*DOMEqF	GAGCAATAAAGCCAAAGGGAG
*Es*DOMEqR	CCCATCAAACATCTTAGGTCGT
*Es*JAKqF	TGTGCGGGAACGGTATCTC
*Es*JAKqR	GTGCGTGGCTGTAGCGTAG
*Es*STATqF	CTCCTGTCCTCGCTTGTTACC
*Es*STATqR	GCCTTGAGCCCTGGTGATA
ALF1 qF	GACGCAGGAGGATGCTAAC
ALF1 qR	TGATGGCAGATGAAGGACAC
ALF2 qF	GACCCTTTGCTGAATGCTTGA
ALF2 qR	CTGCTCTACAATGTCGCCTGA
ALF3 qF	ACGAGGAGCAAGGAAAGAAAG
ALF3 qR	TTGTGCCATAGACCAGAGACTT
Crus-1 qF	GCTCTATGGCGGAGGATGTCA
Crus-1 qR	CGGGCTTCAGACCCACTTTAC
Crus-2 qF	GCCCACCTCCCAAACCTAT
Crus-2 qR	GCAAGCGTCACAGCAGCACT
DWDqF	ACGGGTCGTCAACGAAACTG
DWDqR	GGTCACTGGGTTACCATAGCG
LYSqF	CTGGGATGATGTGGAGAAGTGC
LYSqR	TTATTCGGTGTGTTATGAGGGGT
RNA interference	
si*Es*DOME F	GGAUCUUCCUAUGAAGCUUTT
si*Es*DOME R	AAGCUUCAUAGGAAGAUCCTT

proteins were constructed using the neighbour-joining (NJ) method with 1000 replications in MEGA 6 software.

2.5. Quantitative real-time PCR (qRT-PCR) of EsDOME, EsJAK and EsSTAT

Total RNA was collected from different tissues and hemolymph in crabs challenged by LPS, *V. parahaemolyticus* or *S. aureus*. For quantitative real-time RT-PCR (qRT-PCR) analysis, total RNA (4 μg) was reverse-transcribed using a PrimeScript Real-time PCR Kit (TaKaRa, Shiga, Japan) according to the manufacturer's protocol. Specific primers for *Es*DOME (*Es*DOMEqF and *Es*DOMEqR), *Es*JAK (*Es*JAKqF and *Es*JAKqR), *Es*STAT (*Es*STATqF and *Es*STATqR) and β-actin (β-actin qF and β-actin qR) were designed and synthesised, and expression in different tissues was analysed by qRT-PCR using SYBR Premix Ex Taq (TliRNaseH Plus; TaKaRa, Shiga, Japan) and a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). Reactions were performed at 95 °C for 2 min, followed by 39 cycles at 95 °C for 5 s and 58 °C for 30 s. CFX Manager™ software was used to analyse gene expression data, and relative expression levels were calculated and quantified using the comparative CT ($2^{-\Delta\Delta Ct}$) method [36]. Each sample was analysed three times and qRT-PCR data were assessed by post-hoc Duncan multiple range tests and one-way analysis of variance (ANOVA). Significance was set at $p < 0.05$. For analysis of the expression of *Es*DOME, *Es*JAK and *Es*STAT after bacterial challenge, the primers and reaction conditions were as described above.

2.6. EsDOME RNA interference (RNAi)

For RNAi experiments, siRNAs targeting green fluorescent protein (GFP; control) and *Es*DOME were synthesised by GenePharma (Shanghai, China). Primer sequences are listed in Table 1 siRNAs (5 mg) targeting *Es*DOME and GFP were separately transfected into cultured *E. sinensis* primary hemocytes for 24 h using siRNA-Mate reagent (GenePharma, Shanghai, China) according to the manufacturer's instructions. The method for collecting primary *E.* sinensis hemocytes is described in Section 2.2, and 3×10^6 hemocytes were cultured in each 60 mm dish (Corning, Corning, NY, USA) in 4 mL of specific medium. Total RNA was extracted to determine the RNAi efficiency.

2.7. Western blotting

Western blotting was used to detect STAT phosphorylation with the commercial anti-STAT5 (phospho Tyr694) antibody (Abcam, USA) [16,17], and STATs in total protein extracts were detected with the anti-STAT5 antibody (Abcam, USA). Hemolymph proteins were obtained from crabs challenged with *V. parahaemolyticus* or *S. aureus* using RIPA Lysis Buffer and quantified using the Bradford method. Each sample was separated by 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (CWBio, China) by electroblotting. The membrane was blocked with 5% Non-Fat Powdered Milk (BD Difco, USA) in TBST (1 × TBS buffer containing 0.1% Tween-20) at room temperature on a shaker for 1 h, then incubated at 4 °C overnight with anti-STAT5 (phospho Tyr694) antibody (anti-β-actin antibody) at a dilution of 1:10000. The next day, membranes were washed several times with TBST for 10 min, then incubated with goat anti-rabbit IgG H &L (HRP; ab205718; Abcam) for 1 h at 37 °C. After washing three times with TBST, immunoreactive protein bands were visualised using an Odyssey CLX instrument (Licor, USA).

2.8. Inhibitor injection

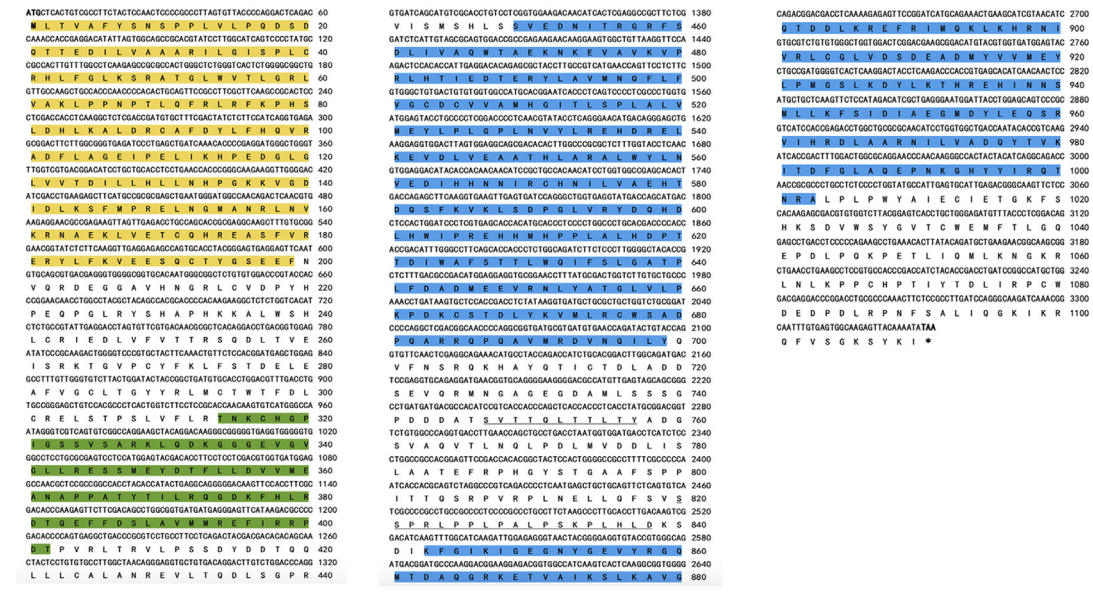
STAT5 inhibitor (573108-10MG, Merck) [16,17] diluted in dimethyl sulfoxide (DMSO) at different concentrations (0.01, 0.1, 1 and 10 μM) was injected into *E. sinensis* primary hemocyte cultures, and primary *E. sinensis* hemocytes were cultured and collected as described in Section 2.2. A total of 3×10^6 hemocytes were cultured in each 60 mm dish (Corning, USA) in 4 mL of specific medium. DMSO injection was used as a control. The level of STAT phosphorylation was determined by western blotting with anti-pSTAT antibody.

2.9. Expression of AMP genes after RNAi silencing of EsDOME and inhibition of EsSTAT protein

RNAi silencing of *Es*DOME was performed as described in section 2.6, and *Es*STAT protein was inhibited as described in section 2.8. After RNAi silencing of *Es*DOME or inhibition of *Es*STAT protein, *E. sinensis* hemocytes were collected to extract RNA at 12 h after *S. aureus* or *V. parahaemolyticus* challenge. qRT-PCR was then performed to measure expression levels of AMP genes *Es*ALF1 [37] (ALF1 qF and ALF1 qR), *Es*ALF2 [32] (ALF2 qF and ALF2 qR), *Es*ALF3 [38] (ALF3 qF and ALF3 qR), *Es*Crus1 [39] (Crus1 qF and Crus1 qR), *Es*Crus2 [30] (Crus2 qF and Crus2 qR), *Es*LYS [40] (LYS qF and LYS qR) and *Es*DWD [41] (DWD qF and DWD qR), as shown in Table 1. All experiments were performed in triplicate.

2.10. Statistical analysis

SPSS software (ver. 11.0) was used for statistical analysis, and statistical significance was calculated by one-way ANOVA and post-hoc Duncan's multiple range tests. Significance was set at $p < 0.05$.



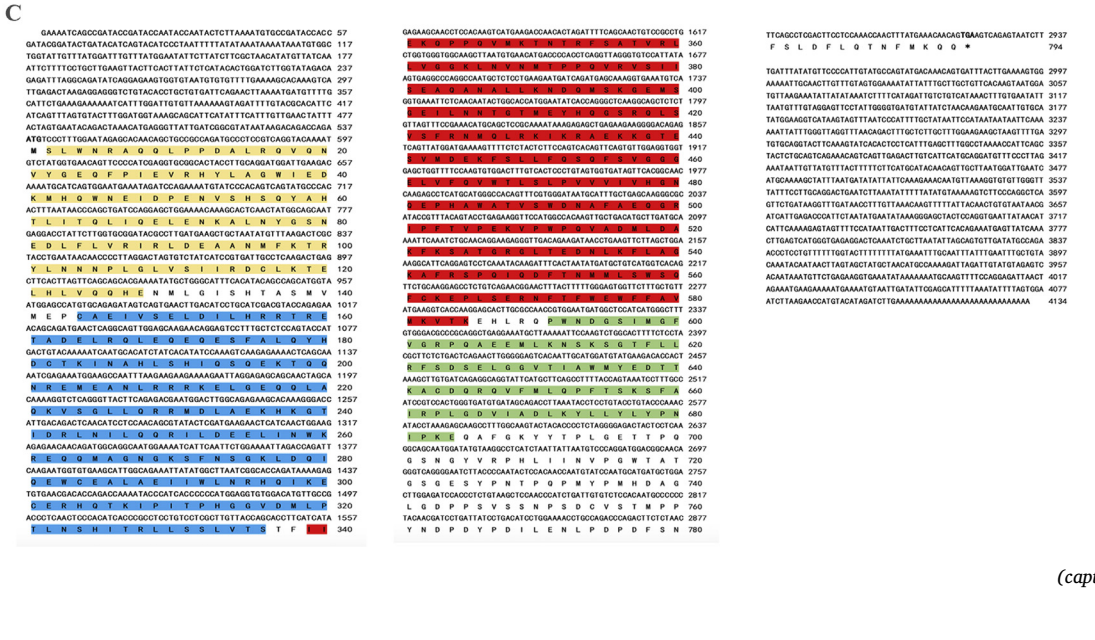


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Fig. 1. Nucleotide and deduced amino acid sequences of *Es*DOME, *Es*JAK and *Es*STAT. Amino acid sequences are shown beneath cDNA sequences. (A) Nucleotide and deduced amino acid sequences of *Es*DOME. The signal peptide region is shaded red, the fibronectin-type-III domains (FN3) are shaded yellow, the transmembrane region is shaded blue, and the low complexity region is underlined. Letters representing start codons (ATG) and stop codons (TAG) are bold. (B) Nucleotide and deduced amino acid sequences of *Es*JAK. The Band 4.1 homology is shaded yellow, the Src homology 2 domain (SH2) domain is shaded green, the tyrosine kinase catalytic domains (TyrKc) are shaded blue, and the low complexity regions are underlined. Letters representing start codons (ATG) and stop codons (TAG) are bold. (C) Nucleotide and deduced amino acid sequences of *Es*STAT. The STAT interaction domain is shaded yellow, the STAT all-alpha domain is shaded blue, the STAT DNA binding domain is shaded red, and the SH2 domain is shaded green. Letters representing start codons (ATG) and stop codons (TAG) are bold. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Cloning and sequence analysis of EsDOME, EsJAK and EsSTAT

The ORF in the *Es*DOME cDNA cloned using the RACE method (GenBank accession number MH294434) is 4200 bp and encodes a 1299 amino acid protein (Fig. 1A). The ORF in the cloned *Es*JAK cDNA (GenBank accession number MH294435) is 3333 bp and encodes a 1110 amino acid protein (Fig. 1B). The full-length *Es*STAT cDNA (GenBank accession number KF471408) is 4134 bp and includes a 2385 bp ORF encoding a 794 amino acid protein, a 537 bp 5 = -untranslated region (UTR) and a 1212 bp 3′-UTR (Fig. 1C). The *Es*DOME protein has a 37 residue signal peptide spanning amino acids (aa) 1–37, a fibronectin-type-III-like domain (aa 143–209), four fibronectin-type-III domains (aa 244–342, 366–450, 576–665 and 681–761), a transmembrane region (aa 878–900) and a low complexity region (aa 985–1002) (Fig. 2A). *Es*JAK contains a Band 4.1 homology domain (aa 1–199), a Src homology 2 domain (aa 314–402), two tyrosine kinase catalytic domains (aa 449–699 and 843–1103) and two low complexity regions (aa 747–757 and 819–838) (Fig. 2B). *Es*STAT includes a STAT interaction domain (aa 2–128), a STAT all-alpha domain (aa 144–336), a STAT DNA binding domain (aa 338–585) and a Src homology 2 domain (aa 590–684) (Fig. 2C). Multiple sequence alignment showed that the three important components in the *E. sinensis* JAK/STAT pathway share low sequence conservation with homologs in other species (Fig. 3).

The three-dimensional models of *Es*DOME (Fig. 2D), *Es*JAK (Fig. 2E) and *Es*STAT (Fig. 2F) were predicted using the online SWISS_MODEL software. In order investigate the evolution of *Es*DOME, we obtained the sequences of DOMEs from crustaceans and insects from GenBank, including tyrosine kinase receptors and tyrosine kinase receptor-like proteins from insects and vertebrates, and IL-6 receptors from other species, and performed phylogenetic analysis. According to the NJ tree,

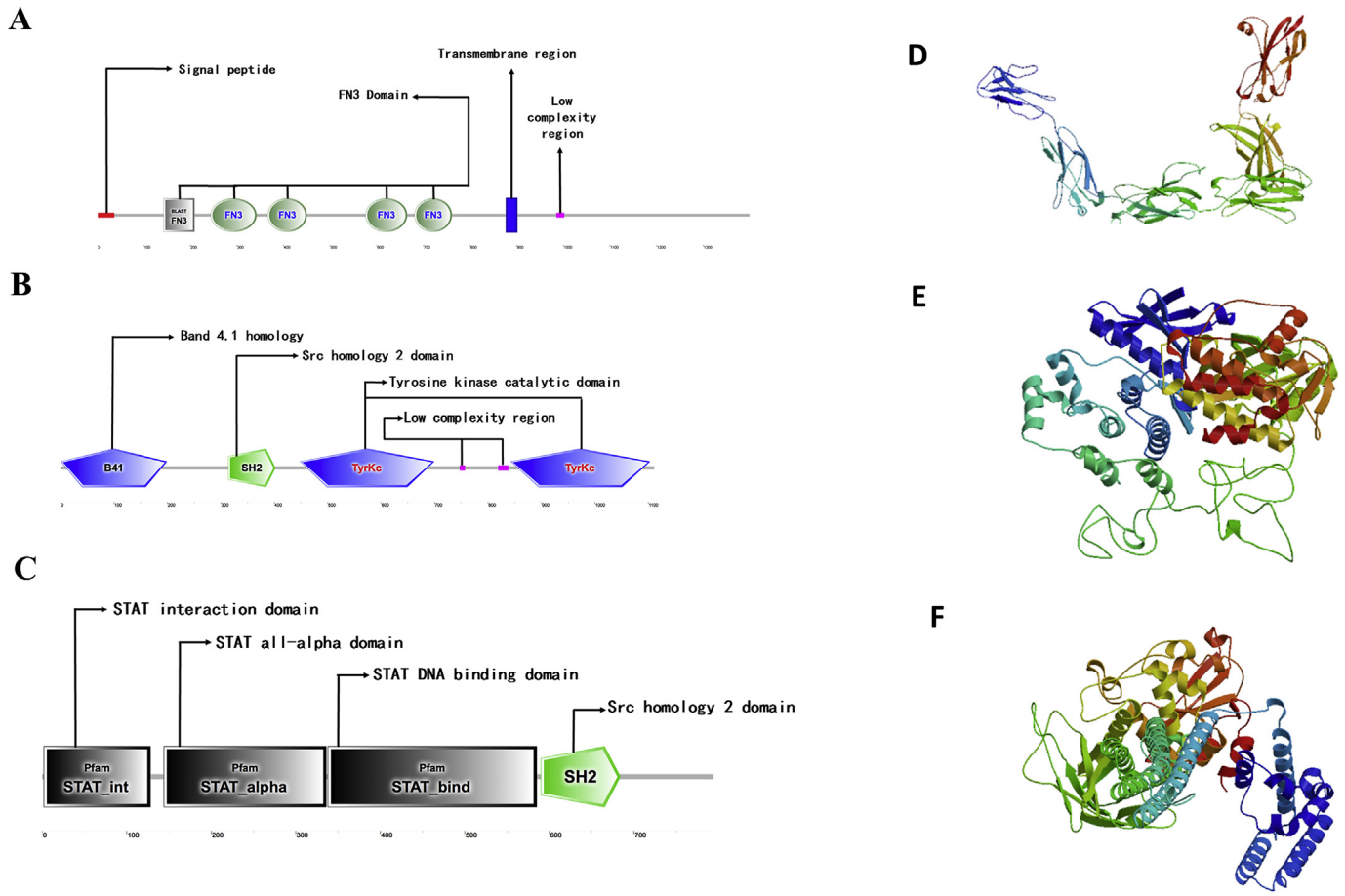

Fig. 2. (A) Domain structure analysis of the putative *Es*DOME protein, which includes a signal peptide, five FN3 domains, a transmembrane region and a low complexity region. (B) Domain structure analysis of the putative *Es*JAK protein, which includes a Band 4.1homology domain, an SH2 domain, two TyrKc domains and two low complexity regions. (C) Domain structure analysis of the putative *Es*STAT protein, which includes a STAT interaction domain, a STAT all-alpha domain, a STAT DNA binding domain and an SH2 domain. (D) Three-dimensional model of the *Es*DOME protein built using SWISS-MODEL based on Protein Data Bank (PDB) ID 3l5h.1.A as template. (E) Three-dimensional model of *Es*JAK built using SWISS-MODEL with PDB ID 4oli.1.A as template. (F) Three-dimensional model of *Es*STAT built using SWISS-MODEL with PDB ID 1yvl.1.A as template.

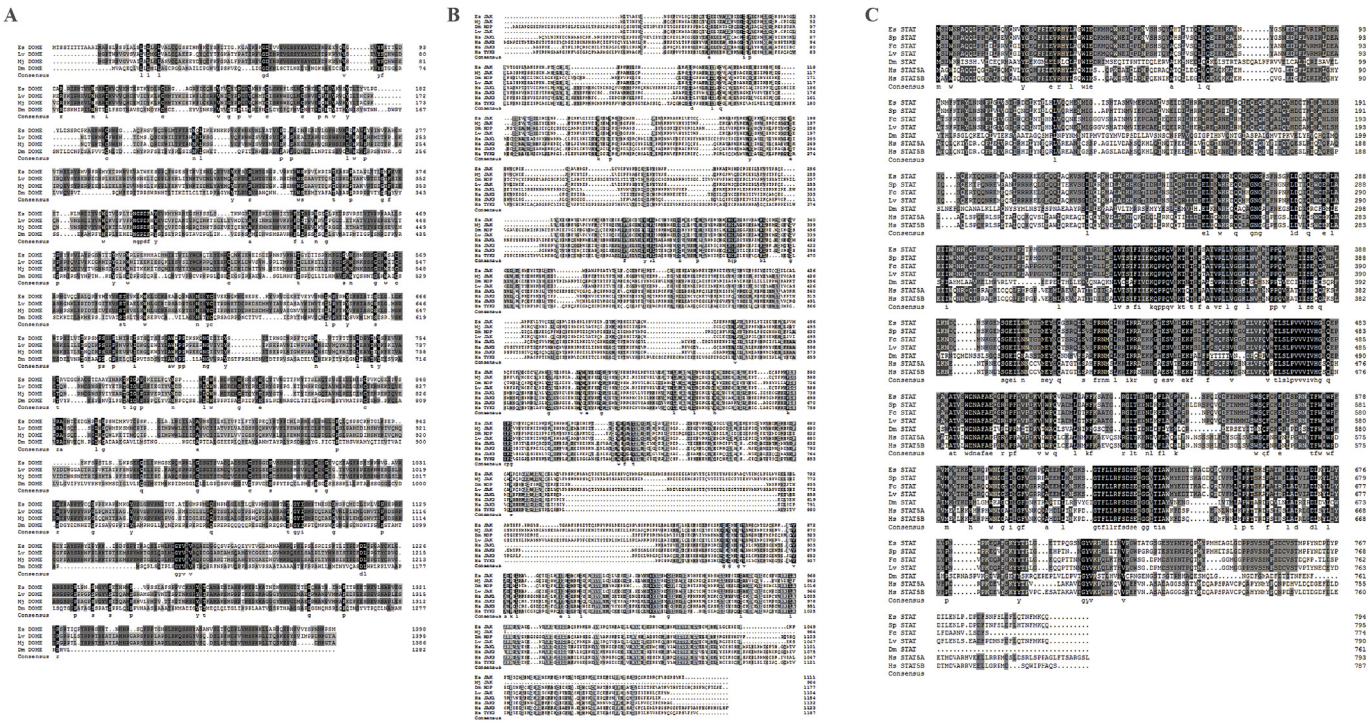

Fig. 3. (A) Multiple sequence alignment of *Es*DOME with other reported Domeless proteins from invertebrates. Sequences and their accession numbers are as follows: *Lv*DOME, *Litopenaeus vannamei*, AGY46351.1; *Mj*DOME, *Marsupenaeus japonicas*, APA16577.1; *Dm*DOME, *Drosophila melanogaster*, AAF49002.1. (B) Multiple sequence alignment of *Es*JAK with other reported JAKs from human and invertebrates. Sequences and their accession numbers are as follows: *Mj*JAK, *Marsupenaeus japonicas*, ANA91281.1; *Dm*HOP, *Drosophila melanogaster*, AAF48035.1; *Lv*JAK, *Litopenaeus vannamei*, AKM12664.1; *Hs*JAK1, *Homo sapiens*, NP_002218.2; *Hs*JAK2, *Homo sapiens*, NP_001309123.1; *Hs*JAK3, *Homo sapiens*, AAC50950.1; *Hs*TYK2, *Homo sapiens*, NP_003322.3. (C) Multiple sequence alignment of *Es*STAT with other reported STATs from human and invertebrates. Sequences and their accession numbers are as follows: *Sp*STAT, *Scylla paramamosain*, AHH29325.1; *Fc*STAT, *Fenneropenaeus chinensis*, ACH70130.1; *Lv*STAT, *Litopenaeus vannamei*, AGT28261.1; *Dm*STAT, *Drosophila melanogaster*, AAC46984.1; *Hs*STAT5A, *Homo sapiens*, NP_001275647.1; *Hs*STAT5B, *Homo sapiens*, NP_036580.2.

all receptor proteins were divided to four groups; Nematoda, Crustacea, Insecota and Verbrata. Therefore, the results of tree analysis were similar to the traditional taxonomic classification (Fig. 4A). Using the same methods, we identified JAKs and STATs in GenBank and performed phylogenetic analysis. JAK proteins were separated into four groups (Insecta, Crustacea, Urochorda and Vertebrata) (Fig. 4B), while STAT proteins were divided into three groups, and *Es*STAT was clustered more tightly with the STAT5 family than other STAT families in vertebrates (Fig. 4C).

3.2. Tissue expression patterns of EsDOME, EsJAK and EsSTAT

Relative *Es*DOME, *Es*JAK and *Es*STAT mRNA expression levels in different tissues were measured by qRT-PCR using β-actin as an internal control. The results showed that *Es*DOME (Fig. 5A), *Es*JAK (Fig. 5B) and *Es*STAT (Fig. 5C) were expressed in a wide variety of tissues, especially immune tissues including gill, intestine, hepatopancreas and heart.

3.3. Expression profiles of EsDOME, EsJAK and EsSTAT in hemocytes following bacterial challenge

qRT-PCR was used to analyse the relative mRNA expression levels of *Es*DOME, *Es*JAK and *Es*STAT in hemocytes following exposure to *S. aureus* or *V. parahemolyticus* as representative Gram-positive and Gram-negative bacteria, respectively. The results showed that *Es*DOME expression was upregulated significantly ($p < 0.05$) compared with controls at 8 h after *S. aureus* challenge (Fig. 6A). In response to *V. parahemolyticus*, expression of *Es*DOME was very significantly upregulated ($p < 0.01$) at 2 h, and maintained at a relatively high level from 2 h to 36 h (Fig. 6B). *Es*JAK expression was very significantly upregulated from 8 h to 36 h after *S. aureus* challenge (Fig. 6C), and from 12 h to 36 h after *V. parahemolyticus* challenge (Fig. 6D). *Es*STAT was upregulated at 4, 12, 24 and 36 h after *S. aureus* challenge (Fig. 6E), 2 and 4, 8, 24 and 36 h after exposure to *V. parahemolyticus* (Fig. 6F).

3.4. Regulation of AMP gene expression by EsDOME

In order to investigate the regulatory function of *Es*DOME on antimicrobial peptides following bacterial challenge, RNAi silencing of *Es*DOME was performed in cultured primary hemocytes. RNAi targeting *Es*DOME was transfected for 24 h and *Es*DOME expression declined by more than 55% compared with the control group transfected with siGFP (Fig. 7A). The mRNA expression levels of *Es*-DWD1, *Es*Lys, *Es*ALF1, *Es*ALF2, *Es*ALF3, Crus*Es*1 and Crus*Es*2 that are closely linked to immunity were measured in *Es*DOME-silenced hemocytes after *S. aureus* or *V. parahemolyticus* challenge. After *Es*DOME silencing and bacterial stimulation, *Es*-DWD1, *Es*Lys, *Es*ALF-1 and *Es*ALF-2 were significantly downregulated following *S. aureus* challenge (Fig. 7B), whereas *Es*-DWD1, *Es*Lys, *Es*ALF-2, *Es*ALF-3 and Crus*Es*2 were downregulated significantly following *V. parahemolyticus* challenge (Fig. 7C).

3.5. Regulation of AMP gene expression by EsSTAT phosphorylation

To investigate the regulatory function of *Es*STAT on antimicrobial peptides following bacterial challenge, a STAT5 inhibitor was used on cultured primary hemocytes to inhibit *Es*STAT phosphorylation. Western blotting analysis showed that the STAT5 inhibitor was most effective at a concentration of 1 μM (compared with the DMSO control) and inhibition lasted for 6 h (Fig. 8A). The mRNA expression levels of *Es*-DWD1, *Es*Lys, *Es*ALF1, *Es*ALF2, *Es*ALF3, Crus*Es*1 and Crus*Es*2 were measured in *Es*STAT-inhibited hemocytes after *S. aureus* or *V. parahemolyticus* challenge. After *Es*STAT inhibition and bacterial

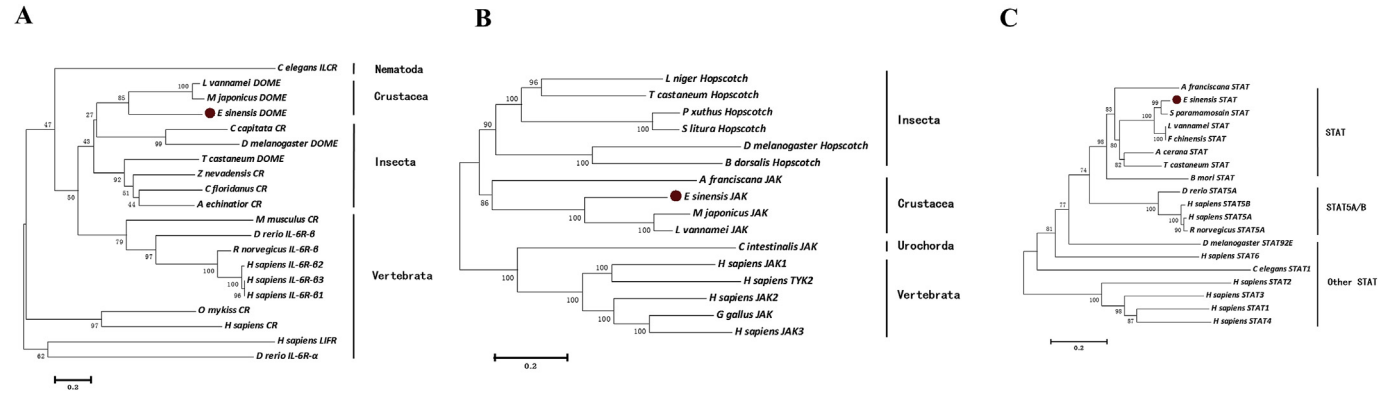

Fig. 4. (A) Neighbour-joining (NJ) phylogenetic analysis of *Es*DOME proteins. The NJ phylogenetic tree of cytokine receptors from other species was constructed using the sequence analysis tool MEGA 6. The red circle delineates *Es*DOME and other sequences, and their accession numbers are as follows: *Caenorhabditis elegans* Interleukin Cytokine Receptor, NP_001022206.1; *Litopenaeus vannamei* Domeless, AGY46351.1; *Marsupenaeus japonicas* Dome, APA16577.1; *Ceratitis capitata* Cytokine Receptor, XP_004518381.1; *Drosophila melanogaster* Domeless, AAF49002.1; *Tribolium castaneum* Domeless, EFA12822.1; *Zootermopsis nevadensis* Cytokine Receptor-like, XP_021937516.1; *Camponotus floridanus* Cytokine Receptor, EFN69830.1; *Acromyrmex echinatior* Cytokine Receptor, EGI63440.1; *Mus musculus* Cytokine Receptor NR10, BAB88745.1; *Danio rerio* Interleukin-6 Receptor-Alpha, NP_001317187.2; *Danio rerio* Interleukin-6 Receptor-Beta, NP_001106976.1; *Rattus norvegicus* Interleukin-6 Receptor-Beta, NP_001008725.2; *Homo sapiens* Interleukin-6 Receptor Beta-3, NP_001177910.1; *Homo sapiens* Interleukin-6 Receptor Beta-2, NP_786943.1; *Homo sapiens* Interleukin-6 Receptor Beta-1, NP_002175.2; *Oncorhynchus mykiss* Cytokine Receptor, CAF05667.1; *Homo sapiens* Cytokine Receptor, AAA93193.1; *Homo sapiens* Leukaemia Inhibitory Factor Receptor, NP_001121143.1. (B) NJ phylogenetic analysis of *Es*JAK proteins. The red circle delineates *Es*JAK and other sequences, and their accession numbers are as follows: *Lasiusniger* Hopscotch, KMQ94378.1; *Tribolium castaneum* Hopscotch, XP_008196394.1; *Papilioxuthus* Hopscotch, KPJ05796.1; *Spodoptera litura* Hopscotch, XP_022831172.1; *Marsupenaeus japonicus* JAK, ANA91281.1; *Drosophila melanogaster* Hopscotch, AAF48035.1; *Bactrocera dorsalis* Hopscotch, JAC54213.1; *Artemia franciscana* JAK, ACJ63722.1; *Litopenaeus vannamei* JAK, AKM12664.1; *Ciona intestinalis* JAK, NP_001071749.1; *Homo sapiens* JAK1, NP_002218.2; *Homo sapiens* JAK2, NP_001309123.1; *Homo sapiens* JAK3, AAC50950.1; *Homo sapiens* TYK2, NP_003322.3; *Gallus gallus* JAK, AAC34195.1. (C) NJ phylogenetic analysis of *Es*STAT proteins. The red circle delineates *Es*STAT and other sequences, and their accession numbers are as follows: *Artemia franciscana* STAT, ACJ63721.1; *Scylla paramamosain* STAT, AHH29325.1; *Litopenaeus vannamei* STAT, AGT28261.1; *Fenneropenaeus chinensis* STAT, ACH70130.1; *Apis cerana* STAT, PBC29396.1; *Tribolium castaneum* STAT, EFA04581.1; *Bombyx mori* STAT, NP_001157388.1; *Danio rerio* STAT5A, NP_919368.2; *Homo sapiens* STAT5B, NP_036580.2; *Homo sapiens* STAT5A, NP_001275647.1; *Rattus norvegicus* STAT5A, NP_058760.1; *Drosophila melanogaster* STAT92E, AAC46984.1; *Homo sapiens* STAT6, AAC67525.1; *Caenorhabditis elegans* STAT1, NP_001122815.1; *Homo sapiens* STAT2, AAH51284.1; *Homo sapiens* STAT3, NP_644805.1; *Homo sapiens* STAT1, ADA59516.1; *Homo sapiens* STAT4, NP_001230764.1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

stimulation, *Es*ALF-1, *Es*ALF-2, *Es*-DWD1 and *Es*Lys were significantly downregulated following *S. aureus* challenge (Fig. 8B), while *Es*ALF-1, *Es*ALF-2, *Es*ALF-3, Crus*Es*2, *Es*Lys and *Es*-DWD1 were downregulated significantly following *V. parahemolyticus* challenge (Fig. 8C).

4. Discussion

Various core components of the JAK/STAT signalling pathway in mammals and invertebrates including human and drosophila have been identified. The basic signal transduction mechanism and a canonical model of the JAK/STAT pathway have also been confirmed [9]. In crustaceans, including shrimp, research on the JAK/STAT pathway has mainly focused on the STAT protein [16,17], and there are few reports on Domeless or JAK in *L. vannamei* [13,15]. Indeed, the present study is the first to identify and characterise JAK/STAT components in *E. sinensis*. Herein, the core components of the JAK/STAT signalling pathway in *E. sinensis* (*Es*DOME, *Es*JAK and *Es*STAT) were cloned, and their functions in antibacterial immune responses were investigated for the first time.

The *Es*DOME protein has five FN3 domains. In *Homo sapiens*, IL-6 receptors acting as receptors in the JAK/STAT pathway are members of the type I cytokine receptor family, and these have only two distinct cytokine binding modules (CBMs). One of these CBM domains has four conserved cysteine residues, and the other has a the Trp-Ser-Xaa-Trp-Ser (WSXWS) five amino acid motif, which participates in activation of receptors to initiate downstream signalling pathways [42]. Some IL-6 receptors, such as leukaemia inhibitory factor (LIF) receptor in mammals and Domeless in Drosophila [9], have several FN3 domains.

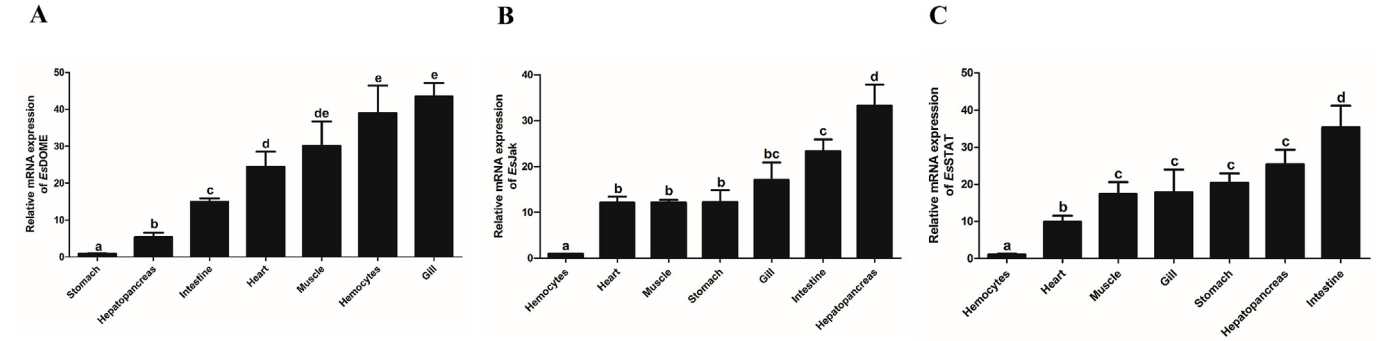

Fig. 5. Tissue-specific expression patterns of *Es*DOME, *Es*JAK and *Es*STAT. Tissue distribution analysis of the expression of (A) *Es*DOME, (B) *Es*JAK and (C) *Es*STAT by qRT-PCR. Expression of β-actin was measured as an internal control. Assays were performed three times, and data are presented as means ± SD. The x-axis represents different tissues in *E. sinensis*, and the y-axis represents corresponding relative expression levels. The same letters above bars indicate that expression levels were not significantly different, while different letters indicate significant differences ($p < 0.05$).

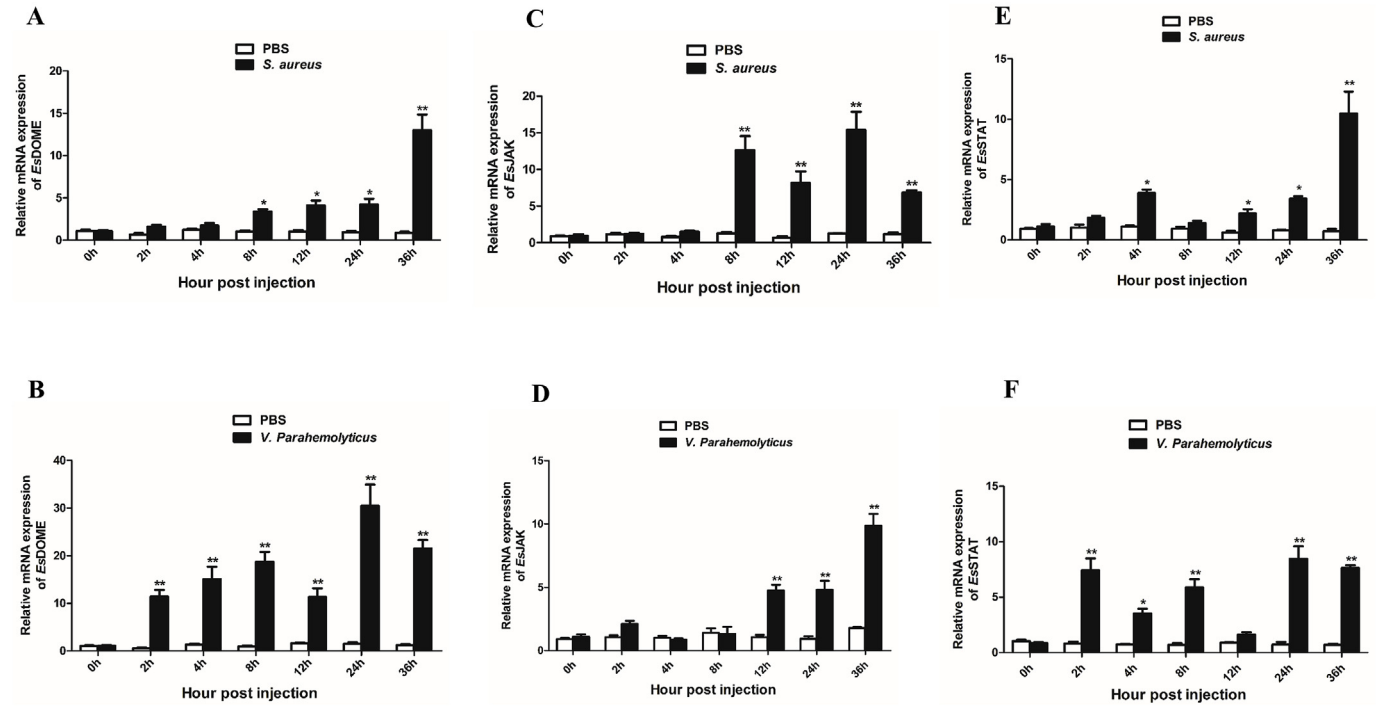

Fig. 6. Expression patterns of *Es*DOME, *Es*JAK and *Es*STAT in hemocytes. Expression of *Es*DOME, *Es*JAK and *Es*STAT in hemocytes after exposure to *S. aureus*, *V. parahaemolyticus* or PBS was measured by qRT-PCR. PBS was used as a control. The x-axis represents the time post-challenge, and the y-axis represents corresponding relative expression levels. Data are means ± SD of triplicate experiments. Statistical significance was determined by one-way analysis of variance (ANOVA) and post-hoc Duncan's multiple range tests. $*p < 0.05$, $**p < 0.01$.

Moreover, *Lv*DOME (DOME in *Litopenaeus vannamei*) also has three FN3 domains, which suggests that DOME as the receptor of JAK/STAT pathway in Chinese mitten crab probably has the similar function with *Lv*DOME [15]. Multiple sequence alignment and phylogenetic tree analysis showed that the *Es*DOME protein is different from IL-6 receptors in mammals and cytokine receptors such as Domeless in insects, but it also shares similar domains such as FN3. Thus, although there are clear differences in structure and function between *Es*DOME and IL-6/cytokine receptors, we cannot exclude the possibility of close functional connections.

JAK tyrosine kinases often include a FERM domain, a Src homology 2 (SH2) domain, a pseudokinase domain, and a TyrKc domain. However, the FERM domain that links the transmembrane receptor cytoplasmic domains and the pseudokinase domain, a dual-specificity protein kinase that negatively regulates cytokine signalling in mammals [9], were not found in *Es*JAK. *Es*JAK does possess a Band 4.1 homology domain, and these specific tyrosine kinase domains are known to play an important role in signalling. *Es*JAK also has an SH2 domain and two TyrKc domains. SH2 domains are known to mediate interactions between receptors, while TyrKc domains are thought to transfer a phosphate group from ATP to a tyrosine residue in a protein, suggesting that *Es*JAK might mediate phosphorylation at specific receptor tyrosine residues, which then serve as docking sites for STATs with the same SH2 domain and/or other signalling molecules. Multiple sequence alignment revealed low homology between *Es*JAK and other JAK family members in invertebrates and mammals. Phylogenetic tree analysis divided sequences into four clades (insects, crustaceans, urochordates and vertebrates). Thus, relationships based on tree analysis correspond to classical taxonomic classification. According to the domain prediction analysis of *Lv*JAK (JAK in *Litopenaeus vannamei*) which also has SH2 domain and TyrKc domain [13] and the result of the NJ phylogenetic tree, *Es*JAK protein is mostly clustered with the crustacean proteins such as *A. franciscana* JAK (*Af*JAK), *M. japonicus* JAK (*Mj*JAK) and *L. vannamei* (*Lv*JAK), and it suggests that the protein JAK plays a similar role in JAK/STAT pathway in crustaceans.

STAT proteins usually consist of a STAT interaction domain, a STAT all-alpha/beta domain, a STAT DNA binding domain, and an SH2 domain, and the STAT interaction domain is thought to facilitate open-ended STAT-STAT interactions [8]. The STAT all-alpha/beta domain is believed to help STAT-STAT dimers enter the nucleus. The DNA-binding domain has an immunoglobulin-like fold similar to the p53 tumour suppressor protein, and the SH2 domain acts as a phosphorylation-dependent switch to control receptor recognition and DNA-binding [9]. Domain prediction revealed that *Es*STAT contains a STAT interaction domain, a STAT all-alpha domain, a STAT DNA binding domain, and an SH2 domain, suggesting that *Es*STAT shares many similarities in structure and function with mammalian STAT family members. Furthermore, multiple sequence alignment revealed high homology between *Es*STAT and homologs in crustaceans *L. vannamei*, *S. paramamosain* and *F. chinensis*. The results of the phylogenetic tree analysis indicated that the *Es*STAT protein is closely related to the *H. sapiens* STAT5 family.

The results of qRT-PCR in different tissues of *E. sinensis* showed that *Es*DOME was expressed at high levels in gill, hemocytes and muscle, while *Es*JAK was expressed highly in hepatopancreas, intestine and gill, and *Es*STAT was expressed at high levels in intestine and hepatopancreas. The relative mRNA expression levels of these three core components of the *E. sinensis* JAK/STAT pathway are similar to those in the crustacean *L. vannamei* [13,15,16]; high expression was observed in gill, hemocytes, hepatopancreas and intestine, all of which are important immune organs in crustaceans.

We therefore speculate that the JAK/STAT signalling pathway in *E. sinensis* is important in host innate immune responses to bacteria, viruses and fungi. To further confirm this, we examined the expression patterns of *Es*DOME, *Es*JAK and *Es*STAT following bacterial stimulation. The results showed that all three components were upregulated in hemocytes following exposure to Gram-positive or Gram-negative bacteria, indicating that the JAK/STAT pathway is closely related to host immune responses in *E. sinensis*. In addition, we found that upregulation of *Es*DOME and *Es*STAT in hemocytes after Gram-negative bacterial

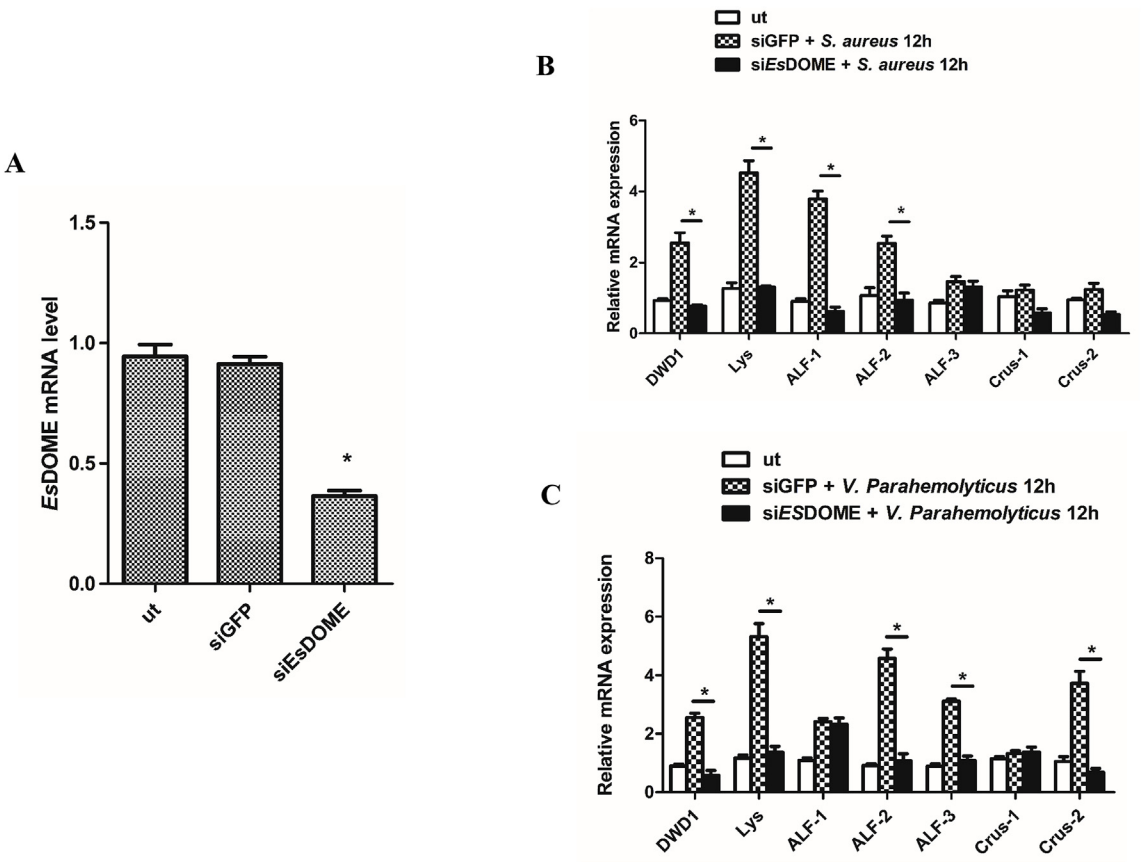

Fig. 7. (A) Efficiency of RNAi silencing of *Es*DOME. qRT-PCR was used to measure *Es*DOME mRNA expression in hemocytes after transfection of GFP siRNA (siGFP) or *Es*DOME siRNA (si*Es*DOME). *Es*DOME mRNA expression in normal hemocytes was used as a control (ut). For qRT-PCR analyses, data for each bar are means ± SD of triplicate experiments. Statistical significance was determined by one-way ANOVA and post-hoc Duncan's multiple range tests. *$p < 0.05$. (B) Effects of *Es*DOME RNAi on expression of antimicrobial peptides (AMPs) in hemocytes challenged with *Staphylococcus aureus*. Following *Es*DOME or GFP knockdown, Hemocytes were challenged with *S. aureus*, and expression levels of seven AMP genes were analysed by qRT-PCR. AMP mRNA expression levels in normal hemocytes were used as controls (ut). Bars represent mean values from three independent repeats, with SD. *$p < 0.05$. (C) Effects of *Es*DOME RNAi on expression of AMPs in hemocytes challenged with *Vibrio parahaemolyticus*.

stimulation was more pronounced and rapid than after exposure to Gram-positive bacteria. These results suggest that the JAK/STAT pathway produces a more sensitive and rapid immune response to Gram-negative bacteria, even though it responds strongly to both Gram-positive and Gram-negative bacteria. In *Litopenaeus vananmei*, the JAK/STAT pathway were also reported to play an important role in response to bacterial infection [13].

The results of the present study revealed that the JAK/STAT pathway regulates the expression of AMPs in *E. sinensis* hemocytes. Domeless acts as a receptor in the JAK/STAT pathway to receive signals from extracellular ligands, and this protein activates the entire signalling pathway in fruit flies [7]. Following RNAi silencing *Es*DOME and bacterial stimulation of hemocytes, we found that expression of some AMPs was downregulated significantly, which suggests that JAK/STAT pathway-mediated regulation of antimicrobial peptide expression in *E. sinensis* involves the Domeless receptor. To confirm this using a different method, we inhibited phosphorylation of *Es*STAT, and AMP expression was similarly downregulated. As in other crustaceans, STATs in crabs are similar to mammalian STAT5 family members [16,17]. In mammals, regulation of cell physiological processes, including survival, proliferation, differentiation and apoptosis, is closely linked to STAT5. However, we speculate that STATs may be translocated into the nucleus, where they can regulate the expression of specific AMP genes. Therefore, we believe that the JAK/STAT pathway regulates the expression of AMPs in *E. sinensis*. In addition, we found a different expression profile of AMPs when challenge with bacteria after Dome-RNAi or injected with STAT inhibitor. *Es*DOME RNA interference (RNAi) in mRNA level and *Es*STAT inhibitor injection in protein level may cause different influences in results [43]. Besides, the inhibition of STAT will only affect the JAK/STAT signalling, because the position of STAT at the end of the entire signalling, and the activation of JAK/STAT signalling will cause the translocation of STAT from cytoplasm to nucleus [8]. However, as the upstream molecules in JAK/STAT signalling, the knockdown of Dome may affect the other signalling that several regulators such as PI3K [44] and Raf [45] and crosstalk with JAK/STAT pathway such as EGFR [46] and PI3K/Akt signalling [44], these signalling pathway and regulators may also influence the expression of AMPs. But the regulators of JAK/STAT pathway and some related crosstalk signalling were not clear in crustacean, so we were not sure which factors affected the expression of antimicrobial peptides via JAK/STAT pathway. These reasons led to a different expression profile of AMPs when challenge with bacteria after Dome-RNAi or injected with STAT inhibitor. Although the JAK/STAT pathway was confirmed to participate in antibacterial immune responses in *E. sinensis*, exactly how signals produced by bacteria are recognised by host cells, and how host cells secrete ligands to activate this pathway, remains unclear. Details of the underlying mechanisms of the JAK/STAT pathway in *E. sinensis* therefore require further in-depth study.

In summary, we cloned and characterised three core components of the JAK/STAT pathway in *E. sinensis*, and conducted bioinformatics, transcriptional and translational analyses. *Es*DOME, *Es*JAK and *Es*STAT were highly expressed in various tissues including gill, hepatopancreas

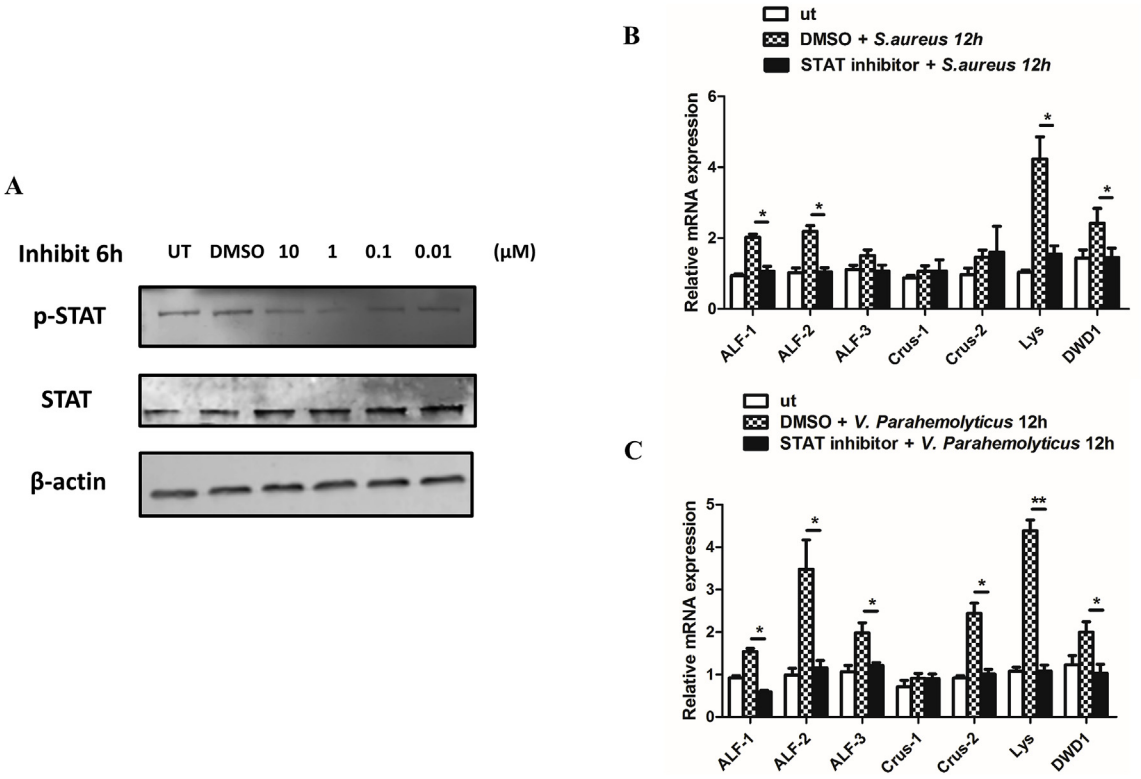

Fig. 8. (A) Efficiency of the STAT inhibitor at different concentrations. Western blotting was used to detect *Es*STAT and p-*Es*STAT protein levels in hemocytes after treatment with different concentrations of the STAT inhibitor (or DMSO as a control). Total *Es*STAT and p-*Es*STAT protein levels in normal hemocytes were treated as ut. The intensities of total *Es*STAT and p-*Es*STAT bands were normalised against β-actin. (B) Effects of inhibiting *Es*STAT on the expression of AMPs in hemocytes challenged with *S. aureus*. Hemocytes treated with STAT inhibitor or DMSO were challenged with *S. aureus*, and expression levels of seven AMP genes were analysed by qRT-PCR. The mRNA expression levels of seven AMPs in normal hemocytes were used as controls (ut). Bars represent mean values from three independent repeats with SD. $*p < 0.05$, $**p < 0.01$. (C) Effects of *Es*DOME RNAi silencing on the expression of AMPs in hemocytes challenged with *Vibrio parahaemolyticus*.

and hemocytes. In addition, all three components were upregulated significantly in hemocytes after exposure to both Gram-positive and Gram-negative bacteria. Moreover, the JAK/STAT signalling pathway positively regulates AMP gene expression, and in doing so, plays an important role in antibacterial immune responses.

Acknowledgements

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