Open

Cellular & Molecular Immunology (2013) 10, 423–436

www.nature.com/cmi

RESEARCH ARTICLE

The shrimp IKK–NF-κB signaling pathway regulates antimicrobial peptide expression and may be subverted by white spot syndrome virus to facilitate viral gene expression

Pei-Hui Wang[1], Zhi-Hua Gu[1], Ding-Hui Wan[1], Bo-Du Liu[1], Xian-De Huang[1], Shao-Ping Weng[1], Xiao-Qiang Yu[2] and Jian-Guo He[1,3]

The IκB kinases IKKα and IKKβ and the IKK-related kinases TANK-binding kinase 1 (TBK1) and IKKε are the master regulators of the NF-κB signaling pathway. Although this pathway has been extensively studied in mammals, less attention has been paid in crustaceans, which have significant economic value. Here, we report the cloning and functional studies of two IKK homologs, LvIKKβ and LvIKKε, from Pacific white shrimp, *Litopenaeus vannamei*. *LvIKKβ* and *LvIKKε* mRNAs are widely expressed in different tissues and are responsive to white spot syndrome virus (WSSV) infection. When overexpressed in *Drosophila S2* cells, LvIKKβ but not LvIKKε activates the promoters of NF-κB pathway-controlled antimicrobial peptide genes (AMPs), such as the *Penaeidins* (*PENs*). In HEK 293T cells, both LvIKKβ and LvIKKε activate an NF-κB reporter. The silencing of *LvIKKβ* or *LvIKKε* using double-stranded RNA (dsRNA)-mediated RNA interference (RNAi) decreases the expression of *L. vannamei* AMPs, including *PENs*, *lysozyme* and *crustins*. Intriguingly, *LvIKKβ*- or *LvIKKε*-silenced *L. vannamei* are resistant to WSSV infection. We hypothesized that successful infection with WSSV requires the activation of the IKK–NF-κB signaling pathway to modulate viral gene expression. We constructed luciferase reporters for 147 WSSV genes. By screening, we found that the *WSSV051*, *WSSV059*, *WSSV069*, *WSSV083*, *WSSV090*, *WSSV107*, *WSSV244*, *WSSV303*, *WSSV371* and *WSSV445* promoters can be activated by LvIKKβ or LvIKKε in *Drosophila* S2 cells. Taken together, our results reveal that LvIKKβ and LvIKKε may participate in the regulation of shrimp AMPs and that WSSV may subvert the *L. vannamei* IKK–NF-κB signaling pathway to facilitate viral gene expression.
Cellular & Molecular Immunology (2013) **10,** 423–436; doi:10.1038/cmi.2013.30; published online 19 August 2013

Keywords: antimicrobial peptide; IKK–NF-κB; innate immunity; *Litopenaeus vannamei*; WSSV

INTRODUCTION

Innate immunity is activated when pathogen signature molecules are recognized by pattern-recognition receptors, such as Toll-like receptors (TLRs), retinoic acid-inducible gene-I-like receptors and nucleotide-binding oligomerization domain-like receptors.[1,2] These signature molecules, which are derived from viruses, pathogenic bacteria, pathogenic fungi and parasitic protozoa, are known as pathogen-associated molecular patterns and include lipopolysaccharide, lipoproteins, single-stranded RNA, double-stranded RNA (dsRNA) and unmethylated CpG-containing DNA.[2–4] In mammals, the recognition of pathogen-associated molecular patterns by TLRs and nucleotide-binding oligomerization domain-like receptors (nucleotide-binding oligomerization domain 1 and nucleotide-binding oligomerization domain 2) activates the NF-κB pathway, which plays a central role in coordinating the expression of pro-inflammatory cytokines and chemokines to eliminate microbial infection by triggering inflammation and recruiting innate and acquired immune cells.[3,4] The activation of NF-κB is achieved by the signal-induced phosphorylation and subsequent degradative polyubiquitylation of the

[1]MOE Key Laboratory of Aquatic Product Safety/State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen University, Guangzhou, China; [2]Division of Cell Biology and Biophysics, School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO, USA and [3]School of Marine Sciences, Sun Yat-Sen University, Guangzhou, China
Correspondence: PH Wang, MOE Key Laboratory of Aquatic Product Safety/State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen University, 135 Xingang Road West, Guangzhou 510275, China.
E-mail: wph326@gmail.com
Or JG He, MOE Key Laboratory of Aquatic Product Safety/State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen University, 135 Xingang Road West, Guangzhou 510275, China.
E-mail: lsshjg@mail.sysu.edu.cn
Received: 14 March 2013; Revised: 30 May 2013; Accepted: 4 June 2013

cytoplasmic inhibitor protein inhibitor of NF-κB alpha (IκBα). IκB kinase alpha (IKKα) and IKKβ mediate the phosphorylation of IκBα and represent a point of convergence for most of the signal transduction pathways that lead to NF-κB activation.[2–4] The degradation of IκBα results in the nuclear translocation of activated NF-κB proteins. In mammals, the recognition of dsRNA by TLR3 and the retinoic acid-inducible gene-I-like receptors (retinoic acid-inducible gene-I and MDA5) also activates the IRF3/7 signaling pathways at the same time as the NF-κB signaling pathway.[1,4,5] The IRF3/7 signaling pathways, which are the key regulators of type I IFN expression, are directly activated in the cytoplasm *via* C-terminal phosphorylation by the IKK-related kinases TANK-binding kinase 1 (TBK1) and IKKε.[5,6] The C-terminal phosphorylation of IRF3 and IRF7 by TBK1 and IKKε promotes their homodimerization and subsequent nuclear translocation, resulting in type I IFN expression.[5,6] IRF7 can also be phosphorylated by IRAK1/IKKα in the TLR7- and TLR9-mediated signaling pathways, resulting in IFNα expression.[2,7]

In *Drosophila*, the Toll and IMD pathways are the best-characterized pattern-recognition receptor pathways that recognize pathogen-associated molecular patterns on the surface of invading bacteria or fungi.[8–10] Although no components of the *Drosophila* Toll and IMD pathways have been identified as virus sensors, both pathways can be activated by viruses, and their activation contributes to the restriction of viral replication.[5,11,12] Upon infection with Gram-negative and certain Gram-positive bacteria, the p100-like NF-κB precursor protein Relish, which is the central component of the IMD pathway, is phosphorylated by IKKβ, activated by proteolytic cleavage, and translocated into the nucleus, where it promotes the expression of immune-related genes, such as antimicrobial peptide genes (AMPs).[8–10,13] Following stimulation by fungi and many Gram-positive bacteria, DIF and Dorsal, which are two p65-like NF-κB proteins of the Toll pathway, are activated by the signal-induced degradation of the IκB-related inhibitor Cactus (*Drosophila* IκBα) and are translocated into the nucleus, where they promote the expression of immune-related genes such as AMPs.[8–10] Like mammalian IκBα, Cactus is phosphorylated upon stimulation; however, its degradation does not require the IKK family proteins, although the IKK complex does act on IκBα in mammals.[14] Currently, it is unknown which kinases can act directly on Cactus. *Drosophila melanogaster* IKKβ functions only in the IMD pathway and not the Toll pathway. Instead of a Cactus (IκBα) kinase, *D. melanogaster* IKKε regulates F-actin assembly by mediating the function of non-apoptotic caspases *via* the degradation of DIAP1 but does not participate in NF-κB activation.[15,16] Whether this phenomenon occurs in other invertebrates and the mechanism of Cactus phosphorylation are still unclear.

Crustaceans include lobsters, crabs, crayfish and shrimp, some of which are of great economic importance, such as cultured penaeid shrimp. Because the production of cultured shrimp is increasing dramatically around the world and increased stress is imposed on aquatic habitats where crustaceans play major ecological roles, crustacean immunology has attracted significant attention in recent years. The Toll and IMD pathways are the major regulators of the immune response in *Drosophila*.[17] Recently, we cloned several members of these two pathways in Pacific white shrimp, *Litopenaeus vannamei*, including LvToll1-3, LvIMD, the p100-like NF-κB precursor protein LvRelish and the NF-κB family protein LvDorsal.[18–21] However, whether the shrimp Toll and IMD pathways also play an essential role in the immune response is still elusive. The rapid and transient expression of AMPs such as the *Penaeidins* (PENs), lysozyme and *crustins* are reported to be pivotal for shrimp defense against microbial infections, and AMP levels are associated with a successful defense against microbial infections by shrimp immune responses.[22] In this study, we cloned two *L. vannamei* IKKs (*LvIKKβ* and *LvIKKε*) and investigated their roles in AMP regulation and viral infection using luciferase assays and dsRNA-mediated gene silencing *in vivo*. We found that successful infection with WSSV may rely on the activation of the IKK–NF-κB signaling pathway, which can significantly induce the promoters of WSSV immediate-early genes, such as *WSSV051*, *WSSV069* (*ie1*) and *WSSV083*.

MATERIALS AND METHODS

Microorganisms

Inocula containing the Gram-negative bacterium *Vibrio alginolyticus* and white spot syndrome virus (WSSV) were prepared as described previously.[23,24] Bacteria were quantified by counting microbial colony-forming units (CFUs) per milliliter on Luria broth agar plates following incubation at 30 °C overnight.

Experimental shrimp

Pacific white shrimp, *L. vannamei* (approximately 8–10 g each for gene expression analysis; approximately 1–2 g each for dsRNA-mediated RNA interference experiments), were purchased from a local shrimp farm in Zhuhai, Guangdong Province, China. The shrimp were cultured in a recirculating water tank system filled with air-pumped seawater (2.5% salinity) at 24–26 °C and fed a commercial diet at 5% of their body weight twice per day. The shrimp were cultured for at least 7 days for acclimation before experiments.

Cloning the cDNA and genomic DNA of LvIKKβ and LvIKKε

In tissue distribution studies of *LvIKKβ* and *LvIKKε*, total RNA (0.5 μg) was isolated from various tissues of healthy shrimp using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and then reverse transcribed into cDNA using a PrimeScript First Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). An *LvIKKε* cDNA fragment was obtained by PCR amplification using degenerate primers (dpIKK-F and dpIKK-R; Table 1) and a cDNA template prepared from shrimp gills. Based on the cDNA fragment and an *LvIKKβ* EST (accession no. CK572131), the full-length cDNAs of *LvIKKε* and *LvIKKβ* were obtained using a RACE-PCR approach as described previously.[18,21] All conditions for RACE-PCR were identical except for the primers (listed in Table 1). Genomic

Table 1 PCR primers used in this study

Primer	*Primer sequence (5′–3′)*
cDNA cloning	
DPLvIKKε-F	GTRCARATGCGIARTTTGAA
DPLvIKKε-R	ACWGCICYYTCATACAT
LvIKKε-5′ RACE1	GCAAGATGTGAAAGCACCAAG
LvIKKε-5′ RACE 2	CCATCACAATCACCTTCCCAC
LvIKKε-3′ RACE 1	GGCTCGCTCTTCAACATTCT
LvIKKε-3′ RACE 2	GCAGGGATGAAACACTTGAGG
LvIKKβ-5′ RACE1	TGTTTAGGGCAGTGAGTGAGC
LvIKKβ-5′ RACE 2	CCCGATGAAGGAAGAACACTG
LvIKKβ-3′ RACE1	CTCACTCACTGCCCTAAACACC
LvIKKβ-3′ RACE2	GCAGCAAGAACCGCACAAC
Genome walking	
LvIKKε-gw1	TAACGCCCTGAAAGACGG
LvIKKε-gw2	GCTGATCCTCGCAGAAATGAC
LvIKKβ-gw1	AAGGTTTCTTCACCCTGTTATT
LvIKKβ-gw2	TCCCTCAACTTCAACCCTCC
dsRNA preparation[a]	
dsGFP-F	AGTGCTTCAGCCGCTACCC
dsGFP-R	GCGCTTCTCGTTGGGGTC
dsGFP(T7)-F	TAATACGACTCACTATAGGAG-TGCTTCAGCCGCTACCC
dsGFP(T7)-R	TAATACGACTCACTATAGGG-CGCTTCTCGTTGGGGTC
dsLvIKKβ-F	GCTGCTGTCCGTTCCTGC
dsLvIKKβ-R	TTTCTCCATTGCGACCTTCA
dsLvIKKβ-F(T7)	TAATACGACTCACTATAGGGC-TGCTGTCCGTTCCTGC
dsLvIKKβ-R(T7)	TAATACGACTCACTATAGGTTT-CTCCATTGCGACCTTCA
dsLvIKKε-F	GAGGCTCGCTCTTCAACATTC
dsLvIKKε-R	TGTTACGCCGACCTCCATAC
dsLvIKKε-F(T7)	TAATACGACTCACTATAGGGA-GGCTCGCTCTTCAACATTC
dsLvIKKε-R(T7)	TAATACGACTCACTATAGGTG-TTACGCCGACCTCCATAC
qPCR analysis	
LvIKKβ-F	ACCACACTTTCCACCTTTGG
LvIKKβ-R	TCCCGATGAAGGAAGAACAC
LvIKKε-F	TTGGCTTCTTTCCAGGACAC
LvIKKε-R	TTTTATGGCTGCCAGGAGTC
LvEF-1α-F	GAAGTAGCCGCCCTGGTTG
LvEF-1α-R	CGGTTAGCCTTGGGGTTGAG
LvPEN2-F	GCATCAAGTTCGGAAGCTGT
LvPEN2-R	ACCCACATCCTTTCCACAAG
LvPEN3-F	CTCTGGCTTGTGGAATGGAT
LvPEN3-R	GCATGGATTCACTTCCTCGT
LvPEN4-F	ATGCTACGGAATTCCCTCCT
LvPEN4-R	ATCCTTGCAACGCATAGACC
Lvlysozyme-F	AAGACACCGAACGATGGAAG
Lvlysozyme-R	TGGGGGACTCGTTCTTTATG
Lvcrustin1-F	GTCGCAGTGCAGGTACTGGT
Lvcrustin1-R	TAGTCGTTGGAGCACGTCTG
Lvcrustin2-F	ATCAGCAGGGGAACAAGAGA
Lvcrustin2-R	CGGACTCGCAGCAATAGACT
Protein expression[b]	
pAc5.1-LvIKKβ-F	AAGGAAAAAAGCGGCCGCAA-CATGGCAGCAGCAGAAGA
pAc5.1-LvIKKβ-R	GCTCTAGACAAGGAAGTTTCAACTGCCTTC
pAc5.1- LvIKKε-F	CGGGGTACCGGAATGGCATTTCTGCGAGGAT
pAc5.1-LvIKKε-R	GCTCTAGACGCAACCTCAGTTTGTAATCTTG
pCMV-LvIKKβ-F	CCGCTCGAGATGGCAGCAGCAGAAGA
pCMV-LvIKKβ-R	GCTCTAGACAAGGAAGTTTCAACTGCCTTC
pCMV-LvIKKε-F	GGAATTCACCATGGCATTTCTGCGAGGAT
pCMV-LvIKKε-R	GCTCTAGACGCAACCTCAGTTTGTAATCTTG

Abbreviations: dsRNA, double-stranded RNA; GFP, green fluorescent protein; PEN, Penaeidins qPCR, quantitative PCR.
[a] T7 RNA polymerase-binding site is underlined.
[b] Primers used in the cellular localization and luciferase reporter assays were the same.

DNA from shrimp muscle was extracted using the Universal Genomic DNA Extraction Kit Ver. 3.0 (TaKaRa, Dalian, China) according to the manufacturer's instructions. The genomic DNA sequences of *LvIKKβ* and *LvIKKε* were obtained by PCR amplification using shrimp genomic DNA and gene-specific primers (Table 1). The genomic DNA sequences adjacent to the 5′ ends of *LvIKKβ* and *LvIKKε* were obtained using a Genome Walker Universal Kit (Clontech, California, USA) as previously described.[25]

Bioinformatic analysis

Nucleotide blast searches of the NCBI database were performed to retrieve IKK-like genes. Multiple sequence alignments were performed using ClustalX 2.0 (http://www.ebi.ac.uk/tools/clustalw2). The amino-acid sequences of LvIKKβ and LvIKKε were deduced using Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de). Neighbor joining phylogenic trees were constructed using MEGA 4.0 (http://www.megasoftware.net/index.html) based on the deduced amino-acid sequences of related genes in typical species. Bootstrap sampling was reiterated 1000 times.

Real-time quantitative PCR

For the tissue distribution analysis, the hemocyte, eyestalk, gill, heart, hepatopancreas, stomach, intestine, nerve, muscle, pyloric caecum and epithelium were collected from healthy shrimp. For immune challenge experiments, healthy shrimp were injected intramuscularly in the third abdominal segment with 100 μl of phosphate-buffered saline (PBS) (control, 137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, and 1.47mM KH_2PO_4, pH 7.3), 100 μl of *V. alginolyticus* inoculum (approximately 2.4×10^6 CFU) or 100 μl of WSSV inoculum (approximately 10^7 copies/shrimp). At 0, 3, 6, 12, 24, 36, 48 and 72 h post-injection (hpi), five shrimp from each group were randomly selected, and the hemocyte, gill, hepatopancreas and intestine were collected for quantitative PCR (qPCR) analysis. Shrimp total RNA was isolated, and qPCR cDNA templates were prepared as previously described.[18,24,26] The expression of *LvIKKβ* and *LvIKKε* in healthy and immune-challenged shrimp was detected using 1 μl of cDNA template, the Master SYBR Green I system and a Light Cycler (Roche, Diagnostics, Mannheim, Germany) with the following cycling parameters: 1 cycle of 95 °C for 30 s followed by 40 cycles of

95 °C for 5 s, 57 °C for 20 s and 78 °C for 1 s. qPCR was performed on three replicates per sample, and three shrimp were analyzed for each sample. The expression of *L. vannamei* elongation factor 1α (*LvEF-1α*) was used as an internal control. Standard curves for *LvIKKβ*, *LvIKKε* and *LvEF-1α* were generated by running triplicate reactions of a 10-fold dilution series (10 different cDNA concentrations). The primer amplification efficiencies for *LvIKKβ*, *LvIKKε* and *LvEF-1α* were 2.032, 2.031 and 2.023, respectively. The relative standard curve method was used for the calculation of fold changes in gene expression.[27]

Plasmid construction

To examine the protein expression in *Drosophila* Schneider S2 cells, pAc5.1/V5-His A (Invitrogen, California, USA) and PCR products amplified using pAcLvIKKβ-F and pAcLvIKKβ-R were double digested, purified, ligated and transformed into competent DH5α cells to select clones for sequencing. The pAc5.1-LvIKKε1 and pAc5.1-LvIKKε2 expression vectors were successfully constructed using the same procedure. We constructed an expression plasmid, pAc5.1-N-GFP, which expresses green fluorescent protein (GFP) in *Drosophila* S2 cells.[23] For protein localization studies in *Drosophila* Schneider S2 cells, the complete LvIKKβ open reading frame (ORF) was inserted into pAc5.1-N-GFP to construct pAc5.1-LvIKKβ-GFP, which expresses a fusion protein comprising full-length LvIKKβ and GFP. pAc5.1-LvIKKε1-GFP and pAc5.1-LvIKKε2-GFP were also constructed using the same methods. For protein expression in HEK 293T cells, the *LvIKKβ*, *LvIKKε1* and *LvIKKε2* ORFs were inserted into pCMV-C-MYC (Beyotime, Shanghai, China) to construct pCMV-LvIKKβ, pCMV-LvIKKε1 and pCMV-LvIKKε2, respectively. The luciferase reporter vectors pGL3-PEN453, pGL3-PEN309, pGL3-PEN4, pGL3-Drs or pGL3-AttA were constructed in previous studies and demonstrated to be regulated predominantly through NF-κB activation.[18,19,21,28–30] Using the same method, 147 WSSV luciferase reporters were constructed as described previously.[25,31] An NF-κB luciferase reporter (Clontech, California, USA) was used to study NF-κB signaling in HEK 293T cells. The pRL-TK luciferase reporter vector (Promega, Madison, WI, USA) was used as an internal standard.

Cell culture, transfection and luciferase assays

Drosophila S2 cells were maintained at 28 °C in Schneider's *Drosophila* medium supplemented with 10% fetal bovine serum (Invitrogen, California, USA) without CO_2. When the culture density reached approximately 6×10^6–20×10^6 viable cells/ml, *Drosophila* S2 cells were passaged onto a new plate at a density of approximately 5×10^5 viable cells/ml. HEK 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 incubator. For luciferase reporter assays, the protein expression plasmid, luciferase reporter plasmid, and pRL-TK *Renilla* luciferase plasmid were cotransfected into *Drosophila* S2 cells or HEK 293T cells that were seeded in 96-well plates 24 h before transfection. *Drosophila* S2 cells were transfected using Effectene Transfection Reagent (Qiagen, Hilden, Germany), and HEK 293T cells were transfected using Lipofectamine 2000 (Invitrogen, California, USA). Cells were harvested 36 h later and lysed for the assessment of protein expression and luciferase activity using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) as previously described.[21,25]

Analysis of LvIKKβ and LvIKKε by confocal microscopy

Drosophila S2 cells were seeded onto coverslips coated with poly-L-lysine in 24-well plates. Approximately 24 h later, the cells were transfected with pAc5.1-N-GFP, pAc5.1-LvIKKβ-GFP, pAc5.1-LvIKKε1-GFP or pAc5.1-LvIKKε2-GFP. At 36 h post-transfection, the cells on the coverslips were washed twice with PBS, fixed using Immunol Staining Fix Solution (Beyotime, Shanghai, China) and stained with Hoechst 33258 Solution (Beyotime, Shanghai, China). The treated cells were observed using a Leica laser scanning confocal microscope.

dsRNA preparation and silencing of LvIKKβ and LvIKKε *in vivo* by dsRNA-mediated RNA interference (RNAi)

dsRNA corresponding to *LvIKKβ*, *LvIKKε* and *GFP* (dsLvIKKβ, dsLvIKKε and dsGFP, respectively) were prepared using the T7 RiboMAX Express Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol, as previously described.[32] Briefly, DNA templates for the production of dsLvIKKβ, dsLvIKKε and dsGFP were amplified by PCR using gene-specific primers (Table 1) with the T7 RNA polymerase-binding site at the 5′ terminus to produce sense and antisense RNA strands separately. Then, the single-stranded RNA was annealed to generate dsRNA. After purification, the dsRNA was quantified and then stored at −80 °C. For dsRNA-mediated RNAi experiments, the experimental group (1–2 g per shrimp) was injected with dsLvIKKβ or dsLvIKKε (1 μg/g shrimp) by intramuscular injection, whereas the control groups were injected with dsGFP or PBS, respectively. To determine silencing effects, gill samples from at least three shrimp per treatment group were collected at 0, 24, 72, 120 and 144 h post-dsRNA injection (hpi), and total RNA was extracted. Total RNA from the gills of the dsRNA-injected *L. vannamei* was reverse transcribed into cDNA for the analysis of *LvIKKβ*, *LvIKKε*, *PEN*, *lysozyme* and *crustin* expression using qPCR as described in the section on 'Real-time quantitative PCR'.

WSSV infection experiments in dsRNA-injected *L. vannamei*

The expression of *LvIKKβ* and *LvIKKε* was significantly reduced in dsLvIKKβ- and dsLvIKKε-injected *L. vannamei*, respectively, compared with the dsGFP-injected control groups (>80%) at most of the time points examined by qPCR analysis. To further investigate the roles of *LvIKKβ* and *LvIKKε* in viral infection, we performed WSSV infection experiments. We infected *L. vannamei* intramuscularly with 100 μl of WSSV inoculum (approximately 10^7 copies/shrimp) at 48 h post-dsRNA injection, and mortalities were recorded.

Statistical analysis

Student's *t*-test was used to compare means between two samples using Microsoft Excel. In all cases, differences were considered significant at $P<0.05$ and highly significant at $P<0.01$.

The data are presented as the mean±standard error (standard error of the mean).

RESULTS

Cloning and sequence analysis of LvIKKβ and LvIKKε

The full-length cDNA of *LvIKKβ* was 2479 bp, with a 2376-bp ORF, a 49-bp 5′ untranslated region and a 54-bp 3′untranslated region (Figure 1a). The sequence of *LvIKKβ* has been deposited in NCBI GenBank under accession no. AEK86518. A sequence analysis indicated that LvIKKβ contains N-terminal protein kinase domains (KDs) and more C-terminally located leucine zipper (LZ) and helix-loop-helix (HLH) motifs but lacks the IKKγ- (also known as NEMO) binding domain (Figure 1b).[6] The full-length LvIKKβ protein exhibits 24.7% and 34.7% identity with *Drosophila* IKKβ and human IKKβ, respectively (Supplementary Figure 2). We obtained two isoforms of *LvIKKε*. The *LvIKKε1* cDNA was 2513 bp, with a 2205-bp ORF, a 17-bp 5′ untranslated region and a 291-bp 3′untranslated region (Figure 1a). Compared with *LvIKKε1*, *LvIKKε2* lacks a 90-bp sequence from 1596 to 1686 bp of *LvIKKε1*, which encodes a 30-amino acid (aa) protein sequence (Figure 1a). The sequences of *LvIKKε1* and *LvIKKε2* have been deposited in NCBI GenBank under accession nos. AEK86519 and AEK86520. LvIKKε contains N-terminal protein KD and C-terminal LZ domains but lacks HLH motifs and the IKKγ-binding domain (Figure 1b).[6] LvIKKε exhibits 36.4% and 35.1% identity with human TBK1 and IKKε, respectively (Supplementary Figure 2). Moreover, LvIKKε shares 23% identity with LvIKKβ.

The genomic sequence of *LvIKKβ* is 7866 bp and harbors 11 exons and 10 introns (Figure 1a). The genomic sequence of *LvIKKε* is 8955 bp (Figure 1a). The *LvIKKε1* cDNA is composed of 14 exons, whereas the *LvIKKε2* cDNA lacks the 11th exon, which encodes a 30-aa protein, compared with *LvIKKε1*, suggesting that *LvIKKε1* and *LvIKKε2* are two alternatively spliced isoforms of *LvIKKε* (Figure 1a). Using Genome Walker, we amplified the genomic regions upstream of the 5′ untranslated regions of *LvIKKβ* and *LvIKKε*. In the 1989-bp region upstream of the *LvIKKβ* 5′ end, NF-κB, SP1, AP-1, GATA and STAT motifs involved in

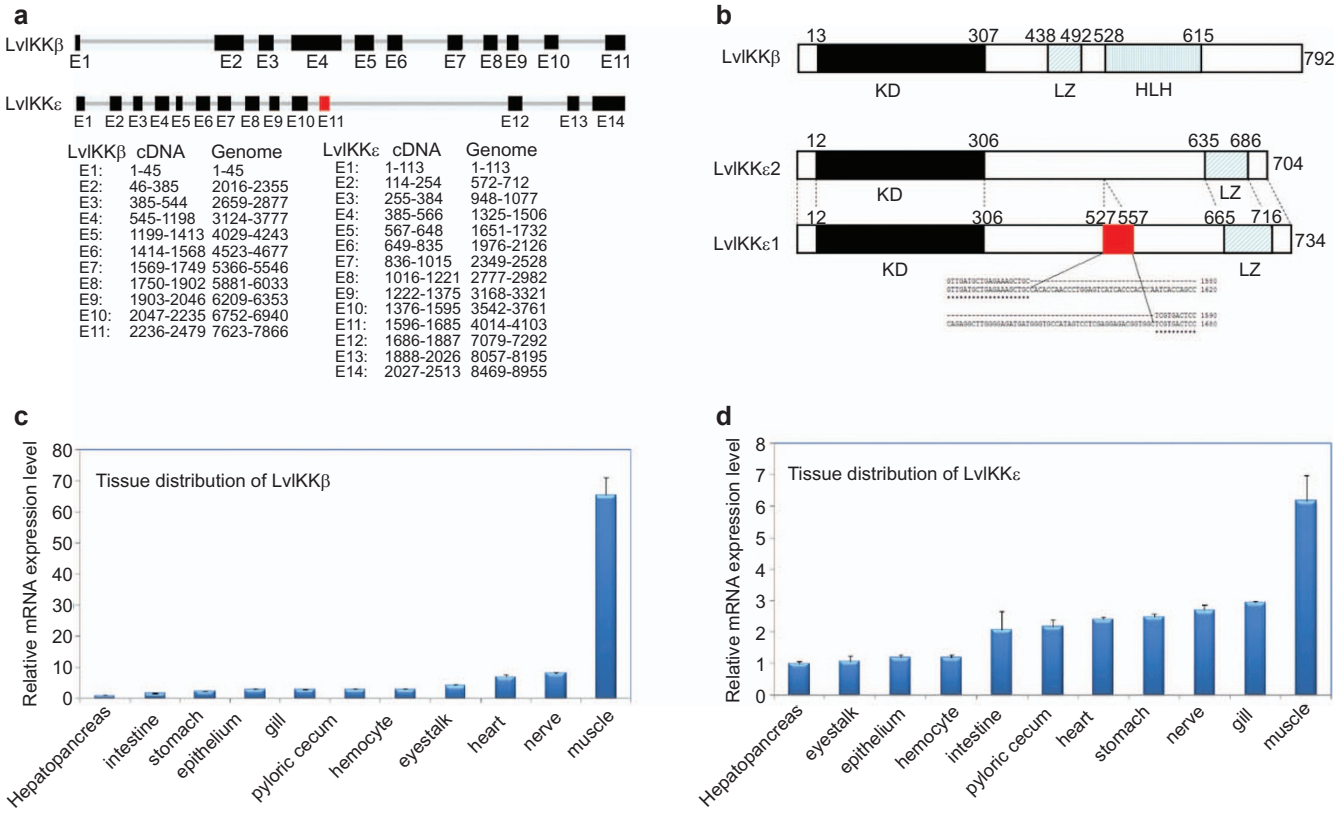

Figure 1 Genomic organization (**a**), domain topology (**b**) and tissue distribution of *LvIKKβ* and *LvIKKε* (**c**). (**a**) Schematic diagrams of the genomic structures of *LvIKKβ* and *LvIKKε* and the splicing isoforms of *LvIKKε*. The numbers represent exons. Exons are depicted as boxes, and introns are depicted as lines. The eleventh exon is red, indicating that it is the eleventh exon of *LvIKKε1* and a partial intron of *LvIKKε2*. (**b**) Schematic representation of the domain topology of LvIKKβ and LvIKKε. LvIKKβ contains N-terminal protein KDs and more C-terminally located LZ and HLH motifs, whereas LvIKKε contains N-terminal protein KDs and a C-terminal LZ domain. (**c**) Tissue distribution of *LvIKKβ* and *LvIKKε* in healthy *L. vannamei*. The hemocyte, hepatopancreas, epithelium, intestine, eyestalk, stomach, gill, heart, pyloric cecum, nerve and muscle were collected from healthy *L. vannamei* to extract total RNA for tissue distribution analysis. The expression was normalized to that of *LvEF-1α* using the relative standard curve method. The expression of *LvIKKβ* and *LvIKKε* in the hepatopancreas was set to 1.0. The data are expressed as the mean fold change (means±s.e., n=3). HLH, helix-loop-helix; KD, kinase domain; LZ, leucine zipper.

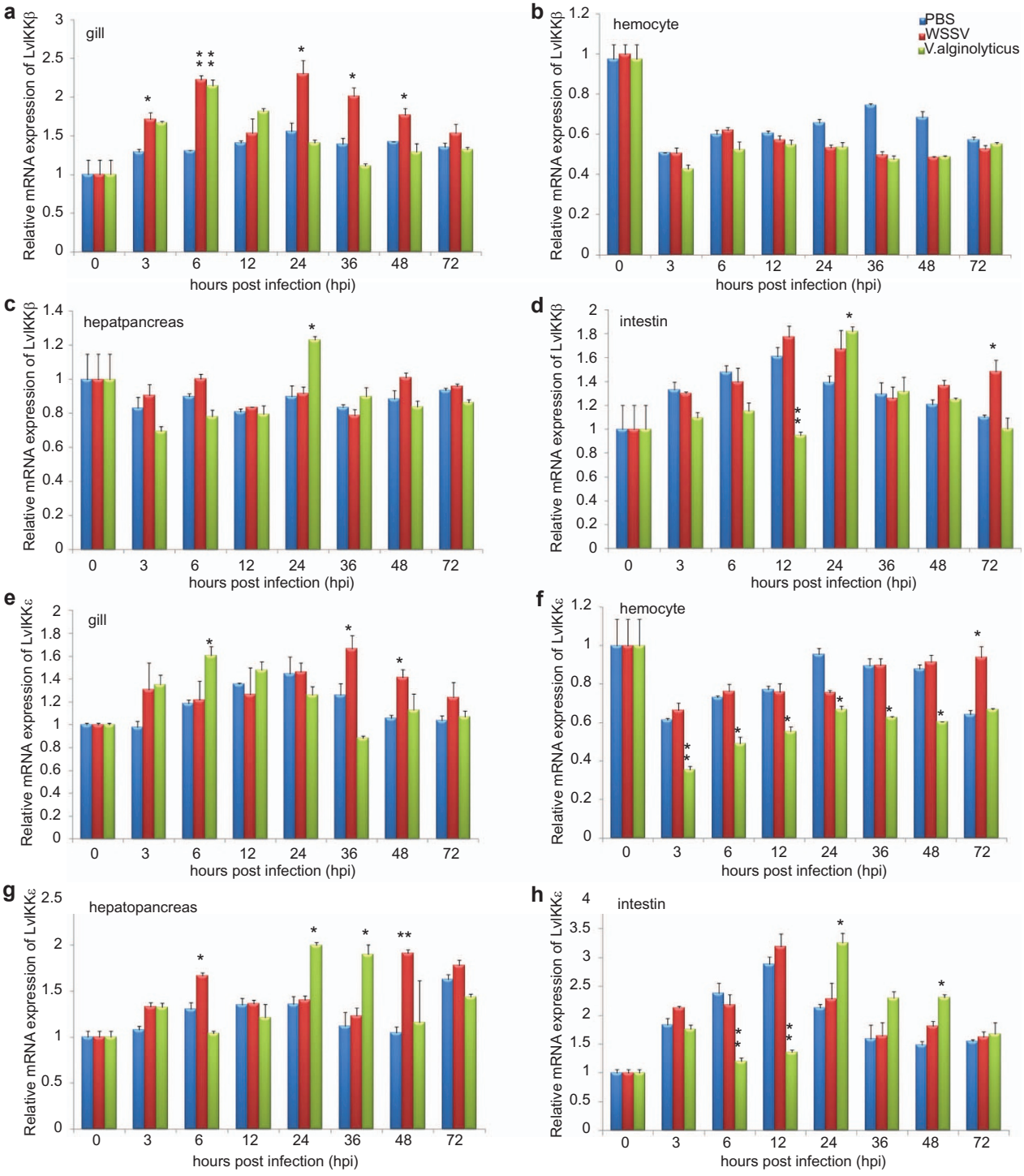

Figure 2 Temporal expression of *LvIKKβ* and *LvIKKε* in the gill, hemocyte, hepatopancreas and intestine after challenge with *V. alginolyticus* or WSSV. Healthy *L. vannamei* were injected intramuscularly at the third abdominal segment with 100 μl of PBS (control group), 100 μl of *V. alginolyticus* (2.4×10^6 CFU) or 100 μl of WSSV inocula (10^7 copies). At 0, 3, 6, 12, 24, 36, 48 and 72 h hpi, five shrimp were randomly selected from each group from which the gill, hemocyte, hepatopancreas and intestine were obtained for qPCR analysis. The expression of *LvIKKβ* and *LvIKKε* in the untreated control group (0 hpi) was set to 1.0. The expression was normalized to that of *LvEF-1α* using the relative standard curve method. qPCR was performed in triplicate for each sample. The data are expressed as the mean fold change (means±s.e., $n=3$) relative to the untreated group (0 hpi). Statistical significance was calculated using Student's *t*-test. Bars with * indicate statistically significant differences ($P<0.05$); bars with ** indicate highly statistically significant ($P<0.01$). CFU, colony-forming unit; hpi, post-injection; PBS, phosphate-buffered saline; qPCR, quantitative PCR; WSSV, white spot syndrome virus.

the transcriptional regulation of immune system genes in arthropods were identified (Supplementary Figure 1a). In the 401-bp region upstream of the *LvIKKε* 5′ untranslated region, GATA, SP1 and TATA box motifs were found (Supplementary Figure 1b).

Phylogenetic tree construction

Phylogenetic analysis of LvIKKβ and LvIKKε showed that LvIKKβ is a member of the IKKα and IKKβ group, whereas LvIKKε belongs to the IKKε and TBK1 group (Supplementary Figure 3). The results also revealed that invertebrates may only possess one member of the IKKα and IKKβ group and one member of the IKKε and TBK1 group.

Tissue distribution of LvIKKβ and LvIKKε in healthy shrimp

In healthy shrimp, *LvIKKβ* was highly expressed in the muscle (65.3-fold), nerve (8.1-fold), heart (7.0-fold), eyestalk (4.3-fold), hemocyte (3.0-fold), pyloric cecum (3.0-fold), gill (2.9-fold), epithelium (2.9-fold), stomach (2.3-fold) and intestine (1.7-fold) compared with mRNA expression in the hepatopancreas (1.0-fold) (Figure 1c). In healthy shrimp, *LvIKKε* was highly expressed in the muscle (6.2-fold), gill (3.0-fold), nerve (2.7-fold), stomach (2.5-fold), heart (2.4-fold), pyloric cecum (2.2-fold), intestine (2.1-fold), hemocyte (1.2-fold), epithelium (1.2-fold) and eyestalk (1.1-fold) compared with mRNA expression in the hepatopancreas (1.0-fold) (Figure 1d).

Expression profiles of LvIKKβ and LvIKKε after microbial challenge

After WSSV infection, the expression of *LvIKKβ* increased in the gill compared with the PBS injection group, but no significant changes were observed in the hemocyte, hepatopancreas or intestine (Figure 2). *LvIKKε* was slightly upregulated in the gill, hemocyte and hepatopancreas but not in the intestine after WSSV infection (Figure 2). After *V. alginolyticus* infection, *LvIKKβ* was upregulated in the gill, hepatopancreas and intestine compared with the PBS injection group, but downregulated in the hemocyte (Figure 2). After *V. alginolyticus* infection, *LvIKKε* was upregulated in the gill, hepatopancreas and intestine and downregulated in the hemocyte (Figure 2).

Cellular localization of LvIKKβ and LvIKKε in Drosophila S2 cells

Using fluorescent imaging by confocal microscopy of the LvIKKβ-GFP and LvIKKε1-GFP fusion proteins, we observed that LvIKKβ-GFP and LvIKKε1-GFP were localized to the cytoplasm and nucleus (Figure 3). However, the green fluorescence of the LvIKKε2-GFP fusion protein was confined to the cytoplasm, suggesting that the 30-aa protein sequence in LvIKKε1, which LvIKKε2 lacks, is a potential nuclear localization signal and is necessary for LvIKKε1 nuclear localization (Figure 3c).

3.6. LvIKKβ but not LvIKKε activates shrimp AMP promoters

In *Drosophila* S2 cells, the expression of LvIKKβ increased the promoter activities of *Drosophila Drosomycin* (*Drs*) and *Attacin* A (*AttA*) approximately 3.00- and 3.09-fold, respectively (Figure 4a). LvIKKβ also induced *Penaeus monodon Penaeidin* promoter activities approximately 2.00- and 3.67-fold for PEN453 and PEN309, respectively, and increased *L. vannamei Penaeidin4* promoter activity approximately 4.82-fold (Figure 4a). However, neither LvIKKε1 nor LvIKKε2 could activate shrimp and *Drosophila* AMP genes in *Drosophila* S2 cells (Figure 4a). These results support the possibility that LvIKKβ serves as a positive regulator of the shrimp NF-κB signaling pathway for AMP activation.

When transfected into HEK 293T cells, LvIKKβ, LvIKKε1 and LvIKKε2 induced NF-κB activity approximately 13.2-, 13.4- and 5.5-fold, respectively, compared with the control group (Figure 4b). In addition, LvIKKε1 induced NF-κB activity to 2.4 times that of LvIKKε2 ($P<0.01$). Therefore, the additional 30-aa nuclear localization signal of LvIKKε1 may be vital for the greater activation of NF-κB luciferase reporters in HEK 293T cells.

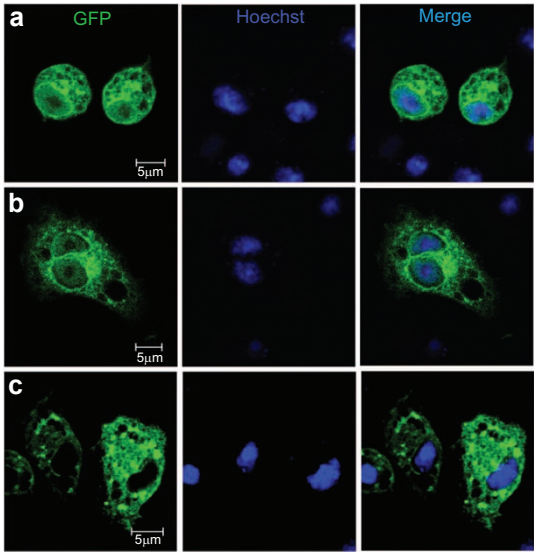

Figure 3 Subcellular localizations of LvIKKβ (**a**), LvIKKε1 (**b**) and LvIKKε2 (**c**) in *Drosophila* S2 cells by confocal microscopy. *Drosophila* S2 cells were transfected with pAc5.1-LvIKKβ-GFP plasmid, pAc5.1-LvIKKε1-GFP plasmid or pAc5.1-LvIKKε2-GFP plasmid. At 36 h post-transfection, the cover slips were washed, fixed, and stained with Hoechst 33258. Localization of recombinant proteins was examined under a Leica laser scanning confocal microscope. (**a**) *Drosophila* S2 cells transfected with pAc5.1-LvIKKβ-GFP showed cytoplasmic and nuclear localization of the LvIKKβ-GFP fusion protein. (**b**) *Drosophila* S2 cells transfected with pAc5.1-LvIKKε1-GFP showed cytoplasmic and nuclear localization of the LvIKKβ-GFP fusion protein. (**c**) *Drosophila* S2 cells transfected with pAc5.1-LvIKKε2-GFP showed cytoplasmic localization of the LvIKKε2-GFP fusion protein. LvIKKε1 contains 30 aa encoded by the eleventh exon of LvIKKε1, suggesting that the 30-aa sequence is necessary for nuclear localization of LvIKKε1. aa, amino acid; GFP, green fluorescent protein.

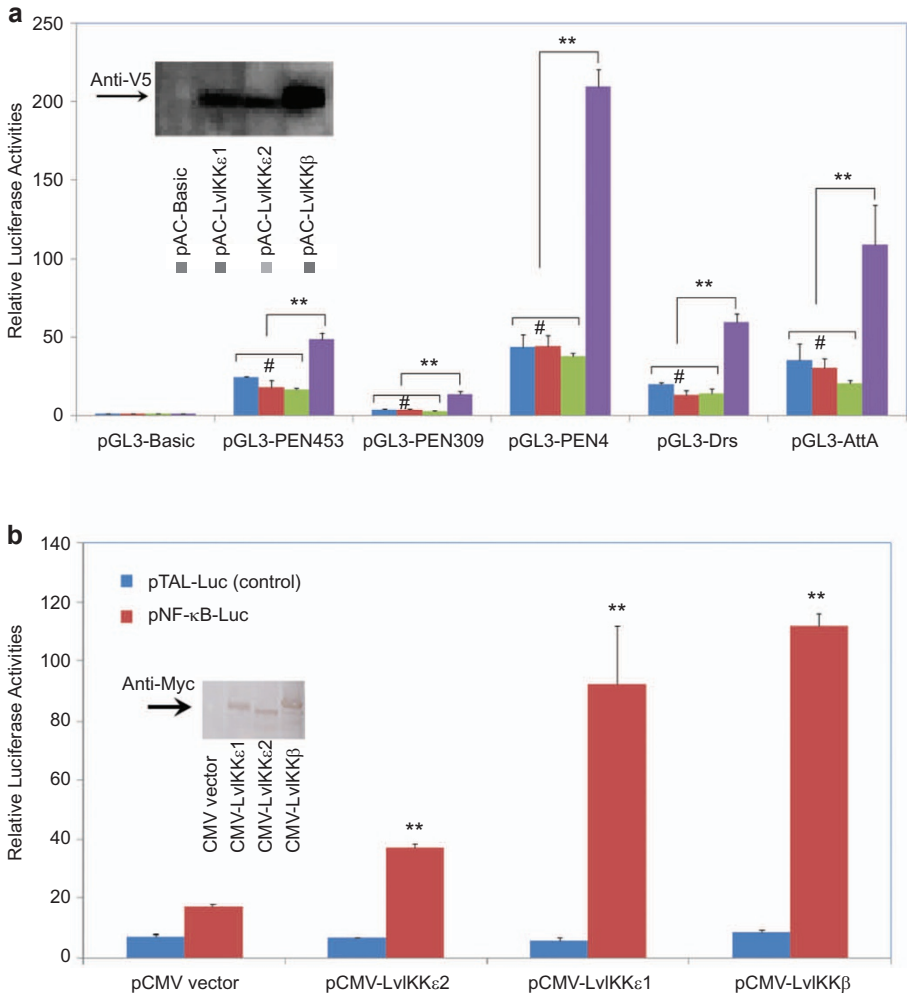

Figure 4 (**a**) The overexpression of LvIKKβ but not LvIKKε activated *Drosophila* and shrimp AMP gene promoters in *Drosophila* S2 cells. *Drosophila* S2 cells were transfected with 0.05 μg of protein expression vector (LvIKKβ, LvIKKε1 or LvIKKε2), 0.05 μg luciferase reporter plasmid (pGL3-Basic, pGL3-PEN453, pGL3-PEN309, pGL3-PEN4, pGL3-Drs, or pGL3-AttA) and 0.005 μg pRL-TK *Renilla* luciferase plasmid (as an internal control; Promega, Madison, WI, USA). (**b**) LvIKKβ and LvIKKε activated NF-κB luciferase reporter in the HEK 293T cells. HEK 293T cells were cotransfected with the pCMV empty vector (70 ng), pCMV-LvIKKε2 (70 ng), pCMV-LvIKKε1 (70 ng) or pCMV-LvIKKβ (70 ng) together with the NF-κB luciferase reporter vector (30 ng) and the internal control *Renilla* expression vector (3 ng). pCMV empty vector and the pTAL luciferase reporter vector (Clontech, California, USA) were used as negative controls (blue bars). At 36 h after transfection, the cells were harvested and analyzed using the Dual Luciferase kit (Promega). The bars indicate the mean±s.d. of luciferase activity (n=3). Statistical significance was calculated by the Student's t-test (not statistical significant $^{\#}P>0.05$; statistical significant $^{*}P<0.05$; highly statistical significant $^{**}P<0.01$). AMP, antimicrobial peptide; CMV, cytomegalovirus; PEN, Penaeidins.

In vivo knockdown of LvIKKβ and LvIKKε by dsRNA-mediated RNAi

To further confirm the roles of *LvIKKβ* and *LvIKKε* in the regulation of shrimp AMPs, dsRNA-mediated RNAi experiments were performed as described previously.[32] The results from the qPCR analysis indicate that we successfully suppressed the expression of *LvIKKβ* and *LvIKKε*. In the gills of dsLvIKKβ-injected *L. vannamei*, the expression of *LvIKKβ* was significantly reduced to 25.7%, 23.4%, 12.2% and 27.6% at 24, 72, 120 and 144 hpi, respectively, of the levels observed in the dsGFP control group (Figure 5a). In the gills of dsLvIKKε-injected *L. vannamei*, the expression of *LvIKKε* was significantly reduced to 16.1%, 23.4%, 12.5% and 10.3% at 24, 72, 120 and 144 hpi, respectively, of the levels observed in the dsGFP control group (Figure 5b).

Decreased AMP expression in LvIKKβ- and LvIKKε-silenced shrimp

Using luciferase assays, we demonstrated that overexpressed LvIKKβ and LvIKKε increase the promoter activities of *LvPEN4* and *P. monodon Penaeidin* (Figure 4a) in *Drosophila* S2 cells. Next, we investigated the effects of LvIKKβ and LvIKKε on the expression of *LvPENs*, *Lvlysozyme* and *Lvcrustins in vivo*. In the gills of *LvIKKβ*-silenced *L. vannamei*, the levels of *LvPEN2*, *LvPEN3*, *LvPEN4*, *Lvlysozyme*, *Lvcrustin1* and *Lvcrustin2* were quantitated using qPCR. We observed that

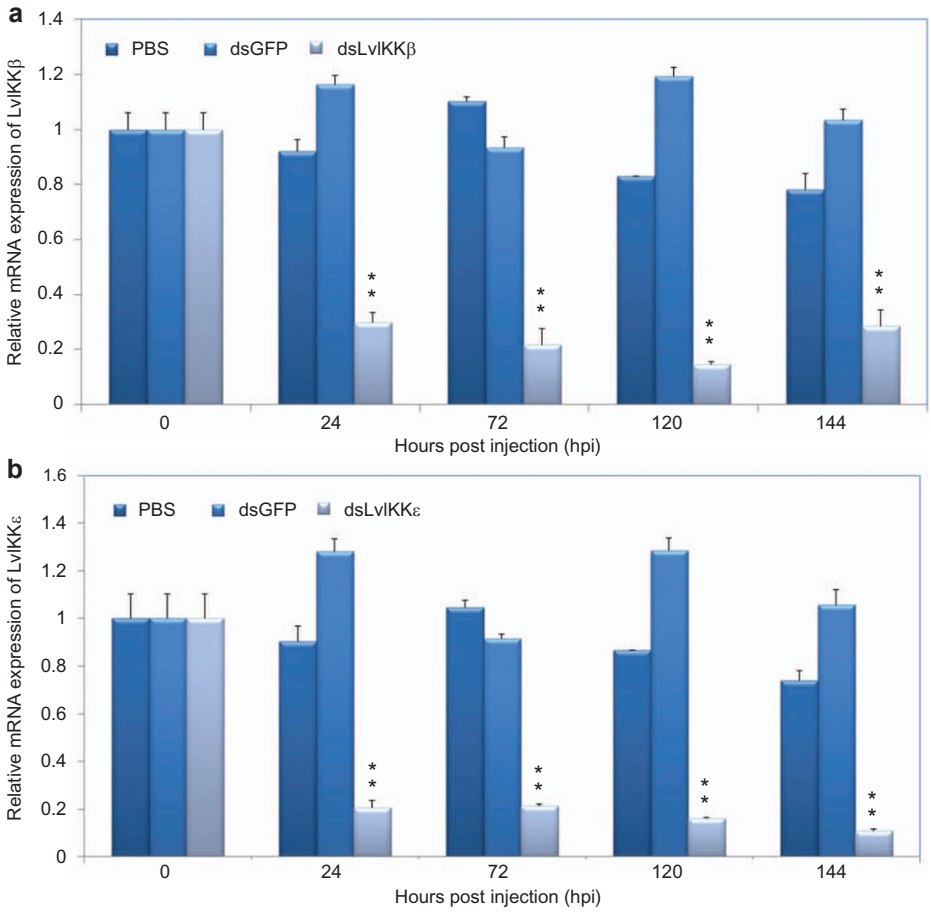

Figure 5 Silencing of *LvIKKβ* (**a**) and *LvIKKε* (**b**) by dsRNA-mediated RNAi. Shrimps were injected with PBS, dsGFP (control), dsLvIKKβ or dsLvIKKε, and the gill was collected at the indicated time points for isolation of total RNA and synthesis of cDNA. The expression levels of *LvIKKβ* and *LvIKKε* were determined by qPCR. qPCR was performed in triplicate for each sample. The data are expressed as the mean fold change (means±s.e., n=3) relative to the untreated group (0 hpi). GFP, green fluorescent protein; hpi, post-injection; PBS, phosphate-buffered saline; RNAi, RNA interference; qPCR, quantitative PCR.

at most of the time points, all of the shrimp AMPs detected, including *LvPEN2*, *LvPEN3*, *LvPEN4*, *Lvlysozyme*, *Lvcrustin1* and *Lvcrustin2*, were significantly downregulated (Figure 6). In the gills of *LvIKKε*-silenced *L. vannamei*, the expression of these shrimp AMPs, including *LvPEN2*, *LvPEN3*, *LvPEN4*, *Lvlysozyme*, *Lvcrustin1* and *Lvcrustin2*, was also significantly decreased (Figure 6).

Knockdown of LvIKKβ and LvIKKε increases survival rates after WSSV infection

WSSV is one of the most common and destructive pathogens in shrimp aquaculture, and shrimp mortality can reach 100% within 3–10 days after infection. To further evaluate the role of *LvIKKβ* and *LvIKKε* in shrimp immune responses, we performed WSSV infection experiments in dsRNA-injected *L. vannamei*. At 48 h after dsRNA injection, *L. vannamei* were infected with WSSV, and mortalities were recorded. We observed that injection of dsGFP, dsLvIKKβ or dsLvIKKε could delay the initial outbreak of WSSV compared with the PBS injection group (Figure 7a). Compared with the dsGFP injection group, dsLvIKKβ- or dsLvIKKε-injected shrimp exhibited significant resistance to WSSV infection from 35 to 98 hpi (Figure 7a).

Screening for viral genes regulated by LvIKKβ and LvIKKε

Because both the *LvIKKβ*- and *LvIKKε*-silenced shrimp were resistant to WSSV infection, we hypothesized that WSSV infection requires the activation of the IKK–NF-κB signaling pathway to facilitate viral gene expression. We constructed luciferase reporters for 147 WSSV genes (Figure 7b). By screening *Drosophila* S2 cells co-transfected with LvIKKβ or LvIKKε, we determined that the *WSSV051*, *WSSV059*, *WSSV069 (ie1)*, *WSSV083*, *WSSV090*, *WSSV107*, *WSSV244*, *WSSV249*, *WSSV303*, *WSSV371* and *WSSV445* promoters can be activated by LvIKKβ or LvIKKε (Figure 7c). Among these, *WSSV051*, *WSSV069 (ie1)* and *WSSV083* are immediate early genes.

DISCUSSION

IKKα/β and TBK1/IKKε are the central regulators of the NF-κB signaling pathway in mammals and represent a point of

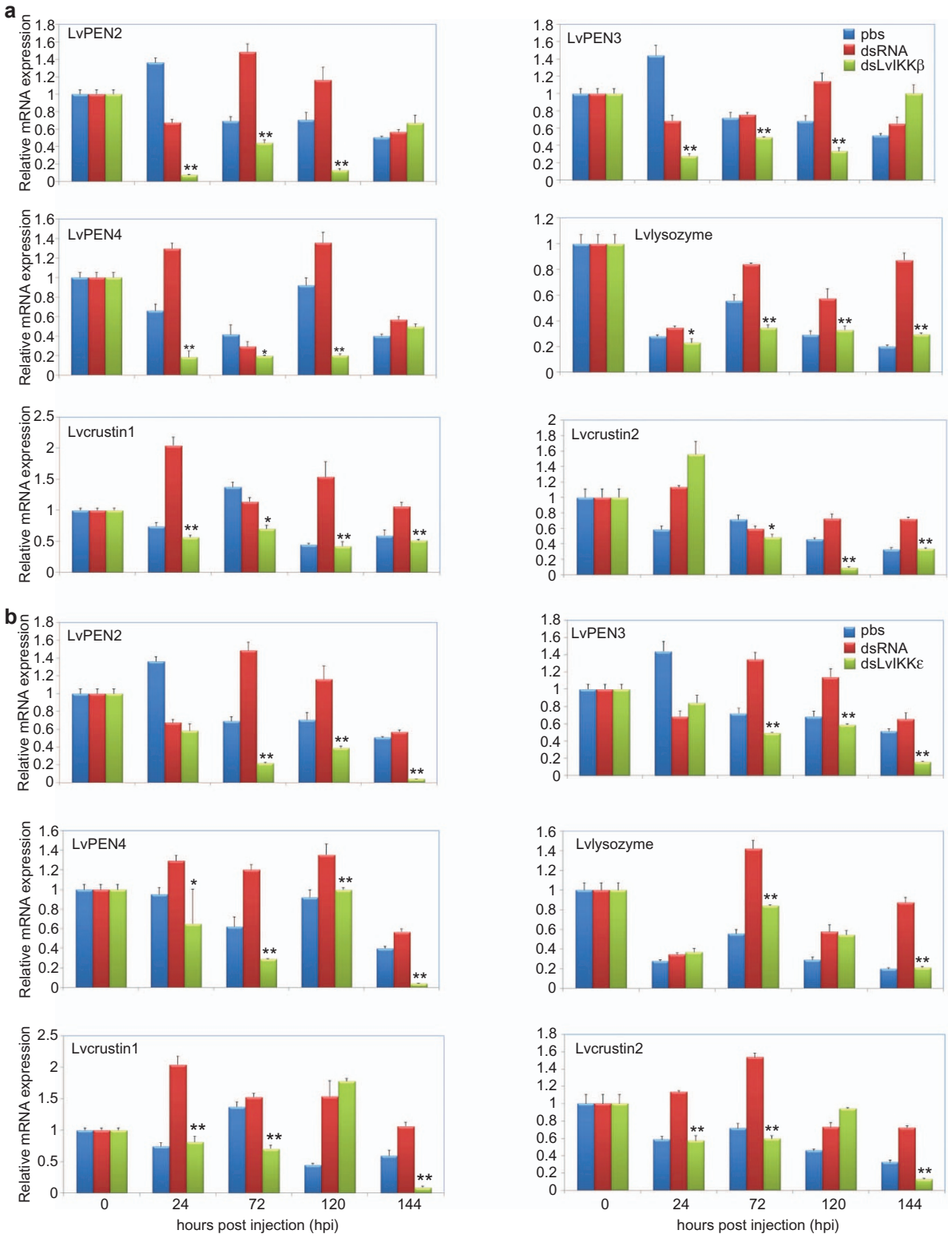

Figure 6 Silencing of *LvIKKβ* or *LvIKKε* led to decrease in expression of shrimp AMPs, including *LvPEN2*, *LvPEN3*, *LvPEN4*, *Lvlysozyme*, *Lvcrustin1* and *Lvcrustin2* in the gill. Shrimps were injected with PBS, dsGFP (control), dsLvIKKβ or dsLvIKKε, and the gill was collected at the indicated time points for isolation of total RNA and synthesis of first-strand cDNA. The expression levels of *LvPEN2*, *LvPEN3*, *LvPEN4*, *Lvlysozyme*, *Lvcrustin1* and *Lvcrustin2* in the gill of dsRNA-injected shrimps were determined using qPCR. qPCR was performed in triplicate for each sample. The data are expressed as the mean fold change (means±s.e., $n=3$) relative to the untreated group (0 hpi). AMP, antimicrobial peptide; dsRNA, double-stranded RNA; hpi, post-injection; PBS, phosphate-buffered saline; PEN, Penaeidins; qPCR, quantitative PCR.

convergence for most of the signal transduction pathways that lead to NF-κB activation.[33] The IKK–NF-κB cascades are also common targets of various viruses, such as the Tax transactivator oncoprotein of HTLV-1, which can directly target IKKα/β and lead to NF-κB activation, resulting in the upregulation of NF-κB signaling pathway-controlled genes.[34] However, in invertebrates, most studies of IKK family proteins have been performed in *D. melanogaster*. In this study, we investigated the roles of LvIKKβ and LvIKKε in shrimp AMP regulation and WSSV infection.

Here, we report two isoforms of IKKε from *L. vannamei*: LvIKKε1 and LvIKKε2. LvIKKε1 possesses an additional 30-aa protein sequence that is absent in LvIKKε2 and other species. Confocal microscopy indicates that this 30-aa protein sequence may be responsible for the cellular localization of LvIKKε1 (Figure 3). Moreover, this 30-aa protein sequence may be responsible for the higher activity (2.4-fold higher) of an NF-κB reporter induced by LvIKKε1 than by LvIKKε2 (Figure 4b). LvIKKβ is a unique invertebrate homolog of mammalian IKKα/IKKβ in *L. vannamei* (Supplementary Figure 3). LvIKKβ also strongly induced NF-κB activity (8.1-fold) in human HEK 293T cells (Figure 4b). In *Drosophila* S2 cells, LvIKKβ but not LvIKKε activated the AMP reporters of *Drosophila* and shrimp, consistent with their induction of NF-κB activity in HEK 293T cells (Figure 4a). The lack of activity of LvIKKε on AMP promoters may be explained by the absence of elements or components of the NF-κB signaling pathway in *Drosophila* S2 cells that are essential for LvIKKε activity.

The expression of *Drosophila* and shrimp AMPs is believed to be primarily controlled by the NF-κB signaling pathway.[10,23,30,35,36] In our previous studies, we showed that other components of the shrimp Toll and IMD pathways, such as LvToll1-3, LvIMD, and the NF-κB family proteins LvDorsal and LvRelish, can activate AMP luciferase reporters.[18–21,25] We also reported that the shrimp NF-κB family proteins LvRelish and LvDorsal can not only activate a shrimp AMP luciferase reporter in *Drosophila* S2 cells, but that they can also bind to putative NF-κB-binding sites in the AMP and *WSSV069* (*ie1*) promoter regions.[19,20,25,31] Here, using dsRNA-mediated gene silencing, we further investigated the regulation of shrimp AMPs by the Toll and IMD pathways. In *LvIKKβ*- or *LvIKKε*-silenced shrimp, *LvPEN2*, *LvPEN3*, *LvPEN4*, *Lvlysozyme*, *Lvcrustin1* and *Lvcrustin2* were significantly reduced (Figure 6). Combined with the activation of AMP luciferase reporters by LvIKKβ and LvIKKε, we propose that the IKK–NF-κB signaling pathway regulates shrimp AMP expression. However, the detailed mechanism by which the NF-κB signaling pathway is activated by LvIKKβ and LvIKKε is still unknown. In the *Drosophila* IMD pathway, IKKβ phosphorylates the p100-like NF-κB precursor protein Relish, and phosphorylated Relish is activated by proteolytic cleavage and translocated into the nucleus where it promotes AMP expression.[37] In future studies, we will investigate whether and how LvIKKβ phosphorylates and activate LvRelish. In the *Drosophila* Toll pathway, the activation of the p65-like NF-κB proteins DIF and Dorsal does not require IKKβ or IKKε.[14] To date, it is still unknown which kinase can act directly on Cactus to liberate DIF and Dorsal from the cytoplasm to the nucleus for AMP expression.[14] This study is the first report that an invertebrate IKKε (LvIKKε) participates in the activation of the NF-κB signaling pathway. Which pathway (Toll or IMD) LvIKKε participates in and how LvIKKε activates this pathway is of great interest. We are also attempting to study whether LvIKKε directly phosphorylates LvCactus and activates LvDorsal.

Many viruses such as HIV-1 hijack and stimulate the host NF-κB signaling pathway as part of their life cycles, diverting NF-κB immune regulatory functions to favor viral replication.[38] The WSSV genome encodes a protein, WSSV449, with similarity to LvPelle (LvIRAK4) of the shrimp Toll pathway.[25] WSSV449 could activate the NF-κB pathway at the same time as LvPelle.[25] Several studies have also reported that WSSV infection can activate the shrimp NF-κB signaling pathway.[25,29,31,39–41] In this study, we found that *LvIKKβ*- or *LvIKKε*-silenced shrimp in which the activities of the NF-κB signaling pathway are attenuated are resistant to WSSV infection (Figure 7a). At the same time, we observed that the activities of the NF-κB signaling pathway in *LvIKKβ*- or *LvIKKε*-silenced shrimp are significantly reduced by measuring the expression level of NF-κB signaling pathway-targeted genes, including *LvPEN2*, *LvPEN3*, *LvPEN4*, *Lvlysozyme*, *Lvcrustin1* and *Lvcrustin2* (Figure 6). Like HTLV-1, HIV-1, Xenotropic murine leukemia virus-related virus, cytomegalovirus (CMV), herpesvirus, hepatitis B virus and Epstein–Barr virus, a successful WSSV infection may also rely on the effective activation of the NF-κB signaling pathway.[38,42] To investigate the potential molecular mechanism for activation, we performed a large-scale screening of 147 WSSV gene promoters (Figure 7b). We found that the overexpression of both LvIKKβ and LvIKKε activates the promoters of several WSSV genes, including *WSSV051*, *WSSV059*, *WSSV069 (ie1)*, *WSSV083*, *WSSV090*, *WSSV107*, *WSSV244*, *WSSV249*, *WSSV303*, *WSSV371* and *WSSV445* (Figure 7c, Supplemental Figure 4). Bioinformatic analysis indicates that there are NF-κB binding sites in some, but not all, of these viral gene promoters, suggesting the direct or indirect activation of their promoters by the IKK–NF-κB signaling pathway. *WSSV051*, *WSSV069* (*ie1*) and *WSSV083* are WSSV immediate-early genes that may be important for activating the expression of other WSSV genes and may be advantageous to the virus infection cycle.[43,44] WSSV069 (ie1) can function as a transcriptional regulator, exhibits transactivation and DNA-binding activities and can take advantage of the shrimp STAT pathway to enhance its own expression.[45,46] The functions of WSSV090, WSSV107, WSSV244, *WSSV*303, *WSSV*371 and *WSSV*445 in viral infection, which are of great interest, are still unknown.

In this study, we cloned *LvIKKβ* and *LvIKKε* and characterized their functions in shrimp AMP regulation and WSSV infection. Our *in vitro* and *in vivo* studies demonstrate that

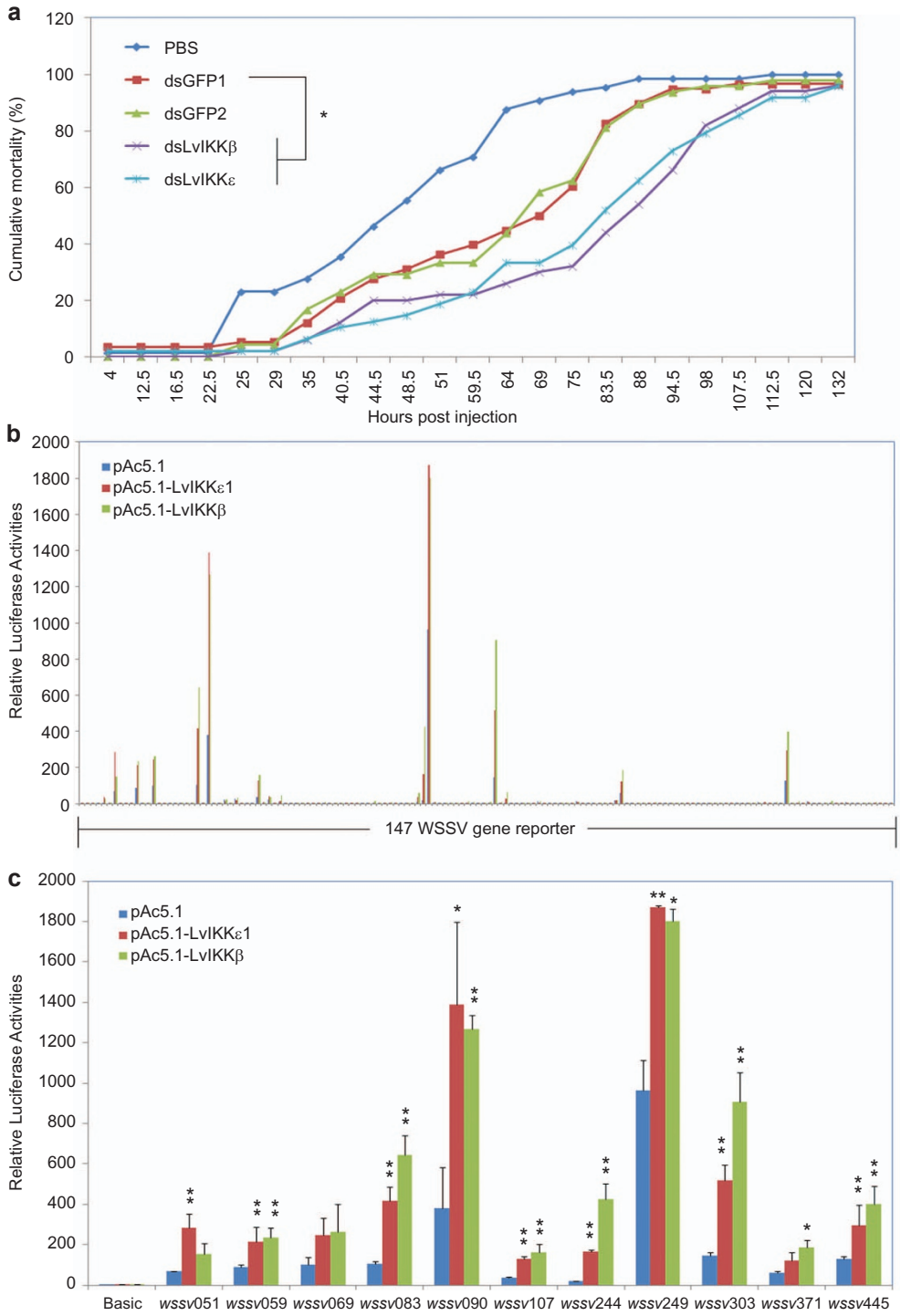

Figure 7 (**a**) Silencing of *LvIKKβ* or *LvIKKε* could delay the outbreak time of WSSV infection. The efficiency of gene silencing in dsLvIKKβ- and dsLvIKKε-injected *L. vannamei* was significant compared with the control groups (>80%) at all the examined time points by qPCR analysis. In the WSSV infection experiments, *L. vannamei* were injected intramuscularly with WSSV inocula (approximately 10^7 copies/shrimp) at 48 h after dsRNA injection. The mortalities of shrimps injected with PBS, dsGFP (control), dsLvIKKβ or dsLvIKKε after WSSV infection were recorded. (**b**) Identification of viral genes activated by LvIKKβ or LvIKKε through large-scale screening. Because both the *LvIKKβ*- and *LvIKKε*-silenced shrimp showed resistant to WSSV infection, we speculated that WSSV infection required activation of the IKK–NF-κB signaling pathway to facilitate viral gene expression. We constructed luciferase reporters for 147 WSSV genes and investigate their activation by LvIKKβ or LvIKKε. (**c**) Promoter activities of *WSSV051*, *WSSV059*, *WSSV069*, *WSSV083*, *WSSV090*, *WSSV107*, *WSSV244*, *WSSV303*, *WSSV371* and *WSSV445* were induced by LvIKKβ and LvIKKε in *Drosophila* S2 cell. dsRNA, double-stranded RNA; GFP, green fluorescent protein; PBS, phosphate-buffered saline; qPCR, quantitative PCR; WSSV, white spot syndrome virus.

both LvIKKβ and LvIKKε can regulate shrimp AMP expression by activating the NF-κB signaling pathway. We also observed that *LvIKKβ*- or *LvIKKε*-silenced shrimp are resistant to WSSV infection, which may be explained by the requirement of IKK–NF-κB signaling pathway activation for WSSV infection. Finally, we also identified the WSSV genes that may be regulated by the IKK–NF-κB signaling pathway through a large-scale viral promoter activity screen. This study extends our knowledge of shrimp AMP regulation and the interaction between the shrimp IKK–NF-κB signaling pathway and WSSV infection.

ACKNOWLEDGEMENTS

This research was supported by National Natural Science Foundation of China under grant No. U1131002, the National High Technology Research and Development Program of China (973 Program) 2012CB114401, Technology Planning Project of Guangdong Province under number 2011A020102002, and China Agriculture Research System CARS-47.

Supplementary Information accompanies the paper on *Cellular & Molecular Immunology* website.

1 Kawai T, Akira S. The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int Immunol* 2009; **21**: 317–337.
2 Kumar H, Kawai T, Akira S. Toll-like receptors and innate immunity. *Biochem Biophys Res Commun* 2009; **388**: 621–625.
3 Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell* 2010; **140**: 805–820.
4 Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; **124**: 783–801.
5 Takeuchi O, Akira S. Innate immunity to virus infection. *Immunol Rev* 2009; **227**: 75–86.
6 Hacker H, Karin M. Regulation and function of IKK and IKK-related kinases. *Sci STKE* 2006; **2006**: re13.
7 Kumar H, Kawai T, Akira S. Pathogen recognition in the innate immune response. *Biochem J* 2009; **420**: 1–16.
8 Ferrandon D, Imler JL, Hetru C, Hoffmann JA. The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. *Nat Rev Immunol* 2007; **7**: 862–874.
9 Hoffmann JA. The immune response of *Drosophila*. *Nature* 2003; **426**: 33–38.
10 Lemaitre B, Hoffmann J. The host defense of *Drosophila melanogaster*. *Annu Rev Immunol* 2007; **25**: 697–743.
11 Sabin LR, Hanna SL, Cherry S. Innate antiviral immunity in *Drosophila*. *Curr Opin Immunol* 2010; **22**: 4–9.
12 Kemp C, Imler JL. Antiviral immunity in *Drosophila*. *Curr Opin Immunol* 2009; **21**: 3–9.
13 Erturk-Hasdemir D, Broemer M, Leulier F, Lane WS, Paquette N, Hwang D *et al*. Two roles for the *Drosophila* IKK complex in the activation of Relish and the induction of antimicrobial peptide genes. *Proc Natl Acad Sci USA* 2009; **106**: 9779–9784.
14 Ganesan S, Aggarwal K, Paquette N, Silverman N. NF-kappaB/Rel proteins and the humoral immune responses of *Drosophila melanogaster*. *Curr Top Microbiol Immunol* 2011; **349**: 25–60.
15 Dubin-Bar D, Bitan A, Bakhrat A, Kaiden-Hasson R, Etzion S, Shaanan B *et al*. The *Drosophila* IKK-related kinase (Ik2) and Spindle-F proteins are part of a complex that regulates cytoskeleton organization during oogenesis. *BMC Cell Biol* 2008; **9**: 51.
16 Kuranaga E, Kanuka H, Tonoki A, Takemoto K, Tomioka T, Kobayashi M *et al*. *Drosophila* IKK-related kinase regulates nonapoptotic function of caspases *via* degradation of IAPs. *Cell* 2006; **126**: 583–596.
17 de Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B. The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. *EMBO J* 2002; **21**: 2568–2579.
18 Wang PH, Liang JP, Gu ZH, Wan DH, Zhu WB, Qiu W *et al*. Molecular cloning, characterization and expression analysis of two novel Tolls (LvToll2 and LvToll3) and three putative Spatzle-like Toll ligands (LvSpz1-3) from *Litopenaeus vannamei*. *Dev Comp Immunol* 2012; **36**: 359–371.
19 Huang XD, Yin ZX, Jia XT, Liang JP, Ai HS, Yang LS *et al*. Identification and functional study of a shrimp Dorsal homologue. *Dev Comp Immunol* 2010; **34**: 107–113.
20 Huang XD, Yin ZX, Liao JX, Wang PH, Yang LS, Ai HS *et al*. Identification and functional study of a shrimp Relish homologue. *Fish Shellfish Immunol* 2009; **27**: 230–238.
21 Wang PH, Gu ZH, Huang XD, Liu BD, Deng XX, Ai HS *et al*. An immune deficiency homolog from the white shrimp, *Litopenaeus vannamei*, activates antimicrobial peptide genes. *Mol Immunol* 2009; **46**: 1897–1904.
22 de Lorgeril J, Gueguen Y, Goarant C, Goyard E, Mugnier C, Fievet J *et al*. A relationship between antimicrobial peptide gene expression and capacity of a selected shrimp line to survive a Vibrio infection. *Mol Immunol* 2008; **45**: 3438–3445.
23 Wang PH, Wan DH, Gu ZH, Deng XX, Weng SP, Yu XQ *et al*. *Litopenaeus vannamei* tumor necrosis factor receptor-associated factor 6 (TRAF6) responds to Vibrio alginolyticus and white spot syndrome virus (WSSV) infection and activates antimicrobial peptide genes. *Dev Comp Immunol* 2011; **35**: 105–114.
24 Wang PH, Wan DH, Pang LR, Gu ZH, Qiu W, Weng SP *et al*. Molecular cloning, characterization and expression analysis of the tumor necrosis factor (TNF) superfamily gene, TNF receptor superfamily gene and lipopolysaccharide-induced TNF-alpha factor (LITAF) gene from *Litopenaeus vannamei*. *Dev Comp Immunol* 2012; **36**: 39–50.
25 Wang PH, Gu ZH, Wan DH, Zhang MY, Weng SP, Yu XQ *et al*. The shrimp NF-kappaB pathway is activated by white spot syndrome virus (WSSV) 449 to facilitate the expression of WSSV069 (ie1), WSSV303 and WSSV371. *PloS ONE* 2011; **6**: e24773.
26 Wang PH, Wan DH, Gu ZH, Deng XX, Weng SP, Yu XQ *et al*. *Litopenaeus vannamei* tumor necrosis factor receptor-associated factor 6 (TRAF6) responds to Vibrio alginolyticus and white spot syndrome virus (WSSV) infection and activates antimicrobial peptide genes. *Dev Comp Immunol* 2011; **35**: 105–114.
27 Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001; **29**: e45.
28 Ho SH, Song YL. Cloning of penaeidin gene promoter in tiger shrimp (*Penaeus monodon*). *Fish Shellfish Immunol* 2009; **27**: 73–77.
29 Li F, Wang D, Li S, Yan H, Zhang J, Wang B *et al*. A Dorsal homolog (FcDorsal) in the Chinese shrimp *Fenneropenaeus chinensis* is responsive to both bacteria and WSSV challenge. *Dev Comp Immunol* 2010; **34**: 874–883.
30 O'Leary NA, Gross PS. Genomic structure and transcriptional regulation of the penaeidin gene family from *Litopenaeus vannamei*. *Gene* 2006; **371**: 75–83.
31 Huang XD, Zhao L, Zhang HQ, Xu XP, Jia XT, Chen YH *et al*. Shrimp NF-kappaB binds to the immediate-early gene ie1 promoter of white spot syndrome virus and upregulates its activity. *Virology* 2010; **406**: 176–180.
32 Wang PH, Gu ZH, Wan DH, Zhu WB, Qiu W, Weng SP *et al*. *Litopenaeus vannamei* sterile-alpha and armadillo motif containing protein (LvSARM) is involved in regulation of penaeidins and antilipopolysaccharide factors. *PloS ONE* 2013; **8**: e52088.
33 Vallabhapurapu S, Karin M. Regulation and function of NF-kappaB transcription factors in the immune system. *Annu Rev Immunol* 2009; **27**: 693–733.
34 Zhi H, Yang L, Kuo YL, Ho YK, Shih HM, Giam CZ. NF-kappaB hyper-activation by HTLV-1 tax induces cellular senescence, but can be alleviated by the viral anti-sense protein HBZ. *PLoS Pathog* 2011; **7**: e1002025.
35 Wang PH, Gu ZH, Wan DH, Zhang MY, Weng SP, Yu XQ *et al*. *Litopenaeus vannamei* Toll-interacting protein (LvTollip) is a

potential negative regulator of the shrimp Toll pathway involved in the regulation of the shrimp antimicrobial peptide gene penaeidin-4 (PEN4). *Dev Comp Immunol* 2013; **40**: 266–277.
36 Kadalayil L, Petersen UM, Engstrom Y. Adjacent GATA and kappa B-like motifs regulate the expression of a *Drosophila* immune gene. *Nucleic Acids Res* 1997; **25**: 1233–1239.
37 Valanne S, Wang JH, Ramet M. The *Drosophila* Toll signaling pathway. *J Immunol* 2011; **186**: 649–656.
38 Hiscott J, Nguyen TL, Arguello M, Nakhaei P, Paz S. Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. *Oncogene* 2006; **25**: 6844–6867.
39 Li F, Yan H, Wang D, Priya TA, Li S, Wang B *et al*. Identification of a novel relish homolog in Chinese shrimp *Fenneropenaeus chinensis* and its function in regulating the transcription of antimicrobial peptides. *Dev Comp Immunol* 2009; **33**: 1093–1101.
40 Zhang S, Li CZ, Yan H, Qiu W, Chen YG, Wang PH *et al*. Identification and function of myeloid differentiation factor 88 (MyD88) in *Litopenaeus vannamei*. *PloS ONE* 2012; **7**: e47038.
41 Li C, Chen YX, Zhang S, Lü L, Chen YH, Chai J *et al*. Identification, characterization, and function analysis of the Cactus gene from *Litopenaeus vannamei*. *PloS ONE* 2012; **7**: e49711.
42 Santoro MG, Rossi A, Amici C. NF-kappaB and virus infection: who controls whom. *EMBO J* 2003; **22**: 2552–2560.
43 Liu WJ, Chang YS, Wang CH, Kou GH, Lo CF. Microarray and RT-PCR screening for white spot syndrome virus immediate-early genes in cycloheximide-treated shrimp. *Virology* 2005; **334**: 327–341.
44 Li F, Li M, Ke W, Ji Y, Bian X, Yan X. Identification of the immediate-early genes of white spot syndrome virus. *Virology* 2009; **385**: 267–274.
45 Liu WJ, Chang YS, Wang HC, Leu JH, Kou GH, Lo CF. Transactivation, dimerization, and DNA-binding activity of white spot syndrome virus immediate-early protein IE1. *J Virol* 2008; **82**: 11362–11373.
46 Liu WJ, Chang YS, Wang AH, Kou GH, Lo CF. White spot syndrome virus annexes a shrimp STAT to enhance expression of the immediate-early gene ie1. *J Virol* 2007; **81**: 1461–1471.