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Transforming growth factor-β-activating kinase 1 and its binding protein 1 participate in the innate immune responses via modulating the IMD—NF—κB signaling in mud crab (*Scylla paramamosain*)

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ABSTRACT

Transforming growth factor-β-activating kinase 1 (TAK1) is essential for diverse important biological functions, such as innate immunity, development and cell survival. In the present study, the homologs of TAK1 and TAK1-binding protein 1 (TAB1) were identified and characterized from mud crab *Scylla paramamosain* for the first time. The full-length cDNAs of *SpTAK1* and *SpTAB1* were 2, 226 bp and 2, 433 bp with 1, 782 bp and 1, 533 bp open reading frame (ORF), respectively. The deduced *Sp*TAK1 protein contained a conserved S_TKc (Serine/threonine protein kinases, catalytic) domain, and the putative *Sp*TAB1 protein possessed a typical PP2Cc (Serine/threonine phosphatases, family 2C, catalytic) domain and a potential TAK1 docking motif. Real-time PCR analysis showed that *SpTAK1* and *SpTAB1* were highly expressed at early development stages, suggesting their participation in crab's development process. Moreover, the expression levels of *SpTAK1* and *SpTAB1* in hepatopancreas were positively stimulated after challenge with *Vibro alginolyticus* and Poly (I:C), implying the involvement of *SpTAK1* and *SpTAB1* in innate immune responses against both bacterial and viral infections. When *SpTAK1* or *SpTAB1* were silenced *in vivo*, the expression levels of two IMD—NF—κB signaling components (*SpIKKβ* and *SpRelish*) and six antimicrobial peptide (AMP) genes (*SpALF1-5* and *SpCrustin*) were significantly reduced, and the bacteria clearance capacity of crabs was also markedly impaired in *SpTAK1* or *SpTAB1* silenced crabs. Additionally, overexpression of *Sp*TAK1 and *Sp*TAB1 in HEK293T cells could markedly activate the mammalian NF-κB signaling. Collectively, our results suggested that TAK1 and TAB1 regulated crab's innate immunity via modulating the IMD—NF—κB signaling. These findings may provide new insights into the TAK1/TAB1-mediated signaling cascades in crustaceans and pave the way for a better understanding of crustacean innate immune system.

1. Introduction

Invertebrates are devoid of adaptive immunity and rely on a rapid and efficient innate immune system to defend themselves from pathogen invasions [1]. Innate immune response is initiated when pattern-recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides, lipoproteins, peptidoglycans and zymosan [2,3]. Upon recognition, PRRs activate specific signaling cascades that manipulate a number of immune effector molecules [3–5]. The Toll signaling pathway and the *immune deficiency* (IMD) pathway are two major pattern-recognition receptor pathways in invertebrates, which regulate the activation of NF-κB and control the expression of antimicrobial peptides (AMPs) [4,6,7].

Transforming growth factor β-activated kinase 1 (TAK1), a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family, was first found to be a mediator in TGF-β superfamily members mediated signaling pathways [8]. Compelling evidence has shown that TAK1 plays a vital role in cell death, differentiation and development, and TAK1 is also a vital regulator in inflammatory responses and innate immunity [9,10]. TAK1 can be activated by a diverse set of stimuli such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and Toll-like receptor (TLR) ligands [11–13]. The activated TAK1 then regulates cell viability and inflammation through activating diverse downstream signaling events such as MAPK (mitogen-activated protein kinase) and

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Table 1
Summary of primers used in this study.

Primers	Sequences (5′ to 3′)
cloning	
TAK1-F	GGCAGTGAAGAAGGTGGAGACAGA
TAK1-R	ACTGTGCCCCTGGGTCTACGGTGT
TAB1-F	ATGCCATCACAAGCCATCAAGC
TAB1-R	AATAGCCAGTCAACACTCAGCT
RACE	
TAK1-3F1	ATGCACCTGTCTGCCATGGATGG
TAK1-3F2	AGATGTGCCTGGTAACACGATTCC
TAK1-5F1	GGCGCTGGCCAGTGATTGAGTC
TAK1-5F2	GGTCAGTAGCAGGTTTGGAGG
TAB1–3F1	ATGGGCTTTACAAGTCTTATCAAG
TAB1–3F2	CCAATACCACTTCCACTGAATCATCT
TAB1–5F1	CTGATGTCATCCCTGAAGGTGA
TAB1–5F2	AAGTAGTTGCCAATACATCGAGTC
UPM	Long:CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT; short: CTAATACGACTCACTATAGGGC
qRT-PCR analysis	
TAK1-qF	GGCAGTGAAGAAGGTGGAGACAGA
TAK1-qR	CAGCCTTGGCACACTGGAACA
TAB1-qF	ATGCCATCACAAGCCATCAAGC
TAB1-qR	CCATCAAACACTCCATAGAGGTAAG
IKKβ-qF	CACGGCTTCTGGCTCTCCTGAT
IKKβ-qR	GATGGCGGGCTTGTAACTTTGCTA
IKKε-qF	GGTCATGCAGTCAAGCGCAAGA
IKKε-qR	TGCACGTGGTTCAGCTGTGA
Relish-qF	AGTGGAACAGTGGTCCAGCTG
Relish-qR	CACCACCACTTCACAAATC
Dorsal-qF	TCATCCCCACAATCTGGTGG
Dorsal-qR	TAAGTGCATCTTCCACGTC
ALF1-qF	TATCCAAGGCACCACAAAGAGGG
ALF1-qR	GTACAGGCACATCACCACCAGCG
ALF2-qF	TGTCGCTCAGGGACTCATCAC
ALF2-qR	GGAGATCACGGGAGAGTGAATG
ALF3-qF	GAACGGACTCATCACACAGCAG
ALF3-qR	CACTTCCTTGTTCTCTTCGCTC
ALF4-qF	CACTACTGTGTCCTGAGCCGC
ALF4-qR	GTCCTCGCCTTACAATCTTCTG
ALF5-qF	CTTGAAGGGACGAGGTGATGAG
ALF5-qR	TGACCAGCCCATTCGCTACAG
Crustin-qF	AAAGCACTATGCGCAAAGAAAACT
Crustin-qR	CACCTTCTGGTAATAGATTATT
β-actin-qF	GCCCTTCCTCACGCTATCCT
β-actin-qR	GCGGCAGTGGTCATCTCCT
Double-strand RNA synthesis	
EGFP-T7F	GGATCCTAATACGACTCACTATAGGATGGTGAGCAAGGGCGAG
EGFP-T7R	GGATCCTAATACGACTCACTATAGGTTACTTGTACAGCTCGTCC
EGFP-F	ATGGTGAGCAAGGGCGAG
EGFP-R	TTACTTGTACAGCTCGTCC
TAK1-T7F	GGATCCTAATACGACTCACTATAGGATTGAAGCCTCCAAGCGGGG
TAK1-T7F	GGATCCTAATACGACTCACTATAGGATGCCGCTGAGCCTCTGTTGTC
TAK1-F	ATTGAAGCCTCCAAGCGGGG
TAK1-R	ATGCCGCTGAGCCTCTGTTGTC
TAB1-T7F	GGATCCTAATACGACTCACTATAGGATGCCATCACAAGCCATCAAGC
TAB1-T7R	GGATCCTAATACGACTCACTATAGGGATAAGACTTGTAAAGCCCATCACTC
TAB1-F	ATGCCATCACAAGCCATCAAGC
TAB1-F	GATAAGACTTGTAAAGCCCATCACTC

NF-κB (nuclear factor-κB) pathways [9,14,15].

The function of TAK1 depends on the formation of a TAK1 complex, which is mediated by association with its binding proteins named TAK1-binding protein1 (TAB1), TAB2 and TAB3 [16–18]. TAB1 constitutively interacts with the N-terminal catalytic domain of TAK1, and directly induces the kinase activity of TAK1 [16]. In mammals, the TAK1/TAB1 complex triggers the phosphorylation and degradation of IκB, which ultimately results in the activation of NF-κB [15,19,20]. In *Drosophila*, the homolog of TAK1 has been identified as a canonical component for the IMD (immune deficiency) signaling pathway [7,21]. When peptidoglycan (PGN) in Gram-negative bacteria are recognized by transmembrane receptors PGRPs (peptidoglycan recognition proteins) [22], the IMD signaling is activated and a signaling complex consisting of the IMD adaptor [23], the adaptor FADD (Fas-associated death domain) and the apical caspase Dredd [24] will be recruited. The adaptor protein IMD, after cleaved by Dredd, then recruits and activates the TAK1/TABs complex which leads to phosphorylation and activation of the IKK complex [25,26]. The IKK complex cleaves the nuclear transcription factor Relish, then the N-terminal fragment of Relish migrates into the nucleus and ultimately trigger the transcription of AMPs [5,27,28]. Nevertheless, the functions and regulatory mechanism of TAK1 and TABs remain largely unknown in crustaceans. Up to now, TAK1, TAB1 and TAB2 have only been identified and characterized from Pacific white shrimp *Litopenaeus vannamei*, and overexpression of

*Lv*TAK1, *Lv*TAB1 or *Lv*TAB2 could significantly stimulate the expressions of AMP genes in *Drosophila* S2 cell line [29–31]. However, whether TAK1 and TABs are involved in innate immune responses as well as the detailed molecular mechanisms underlying their interaction with the IMD signaling pathway are still elusive in crustaceans.

The mud crab *Scylla paramamosain* distributes throughout the southeast Asian coastal areas, and is a commercially important mariculture species in China [32]. Diseases break out frequently in recent years and bring huge economic losses in crab aquaculture. Accordingly, an improved understanding of the innate immunity in crabs might contribute to develop better strategies for the prevention and control of crab diseases. In the present study, we firstly identified the homologs of TAK1 and its binding protein TAB1 in *S. paramamosain*, and investigated their functional roles in crab's innate immunity. Our results demonstrated that *Sp*TAK1 together with *Sp*TAB1 could modulate IMD−NF−κB signaling and regulate the expression of AMP genes. These findings might pave a way for further understanding of the network for TAK1/TAB1-mediated signaling cascades as well as their participation in the innate immune responses in *S. paramamosain* and even in crustaceans.

2. Materials and methods

2.1. Experimental animals

Healthy mud crabs (*S. paramamosain*) weighing 75 ± 5 g were obtained from Sanmen Bay, Zhejiang Province (China) and maintained individually in PVC buckets supplied with continuously aerated artificial seawater (salinity 18‰) at 26 ± 2 °C. During the acclimatization, crabs were fed with fresh manila clam *Ruditapes philippinarum* daily until three days before experiments. The embryos and larvae of *S. paramamosain* were sampled from a breeding base in Jiangsu Province (China) and preserved at −80 °C until further analysis.

2.2. RNA extraction and cDNA synthesis

Samples of mud crabs were collected, immediately freezed with liquid nitrogen and stored at −80 °C until taken for RNA extraction. Total RNA was extracted using RNAiso Plus (Takara, Japan) reagent following the manufacture's protocol. RNA integrity and purity were analyzed by RNA electrophoresis and NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). To synthesis cDNA for PCR and quantitive real-time PCR (qRT-PCR), RNA samples were treated with genomic DNA Eraser, then reversely transcribed into cDNA using PrimeScript™ RT reagent Kit (Takara, Japan).

2.3. PCR amplification and cloning

To obtain the cDNA segments of *SpTAB1* and *SpTAK1*, primers were designed based on the conserved sequences of *TAB1* and *TAK1* genes from other species obtained from NCBI database (Table 1). 500 ng high-quality RNA extracted from hepatopancreas was used as the template and reversely transcribed into cDNA using PrimeScript™ RT reagent Kit (Takara, Japan). The following program was applied for PCR amplification: denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 2 min, and a final extension at 72 °C for 10 min. The obtained PCR products were separated by 1.0% (w/v) agarose gels, recovered with Gel Recovery Kit and cloned into the pMD19-T plasmids. The recombinant plasmids were screened, characterized by PCR amplification and further confirmed by DNA sequencing.

2.4. Rapid amplification of cDNA ends (RACE)

To synthesize first-strand cDNA for RACE reactions, 1 μg high-quality RNA extracted from hepatopancreas was used as the template, and reversely transcribed into cDNA using the SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) according to the manufacture's instructions. For 3′-RACE and 5′-RACE PCR amplification, gene specific primers combined with a universal primer (UPM) were used for nested PCR amplification assay. PCR products were isolated, purified, cloned, and sequenced as described above. Sequences of gene specific primers were listed in Table 1.

2.5. Bioinformatics analysis

Program BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to compare the obtained sequences in the sequence database. DNAStar software was utilized to find the open reading frames (ORFs) of DNA sequences and translate them into amino acids. The protein functional domains were predicted by CDS (Conserved Domain Search) of NCBI (http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi), and Simple Modular Architecture Research Tool (SMART) on SMART website (http://smart.embl-heidelberg.de/). Multiple sequence alignments were generated using the ClustalW program (http://www.genome.jp/tools/clustalw/). Phylogenic trees were constructed via the neighbor-joining (NJ) method using MEGA 5.0 software.

2.6. The quantitive real-time PCR analysis

The expression levels of target genes were determined by qRT-PCR using SYBR Premix Ex Taq™ II kit (Takara, Japan). The amplification of qRT-PCR was carried out according to the following program: first denatured at 95 °C for 30 s, then followed by 45 cycles of 95 °C for 5 s and 60 °C for 30 s, and a final dissociation cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s qRT-PCR amplifications were performed in triplicates for each sample. The relative mRNA expression level was calculated using the Livak ($2^{-\Delta\Delta Ct}$) method [33]. The housekeeping gene *β-actin* was used as the internal control, and the gene-specific qRT-PCR primers were listed in Table 1.

2.7. Immune challenges with Vibro alginolyticus and Poly (I:C)

V. alginolyticus, a common pathogenic bacteria in crab diseases [34], was inoculated on a thiosulfate-citrate-bile-sucrose (TCBS) agar plate, and cultured in LB liquid medium at 28 °C for 12 h, then collected by centrifugation at 3, 000 g for 10 min, and resuspended in PBS to 1.5×10^7 CFU/mL concentration. The virus analogue Poly (I:C) (sigma, USA) was dissolved in PBS to 1 mg/mL concentration. Same volume (100 μL) of *V. alginolyticus*, Poly (I:C) and PBS solution was respectively injected into crabs through the base of fourth paraeiopod. The PBS group was served as the control group. After 0 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h of injection, hepatopancreas of six crabs from each group was sampled for RNA extraction and qRT-PCR assay.

2.8. Knockdown of SpTAB1 and SpTAK1 in vivo by dsRNA interference

Gene-specific primers were designed to obtain DNA templates containing a T7 promotor (Table 1). Double-stranded RNA (dsRNA) for *SpTAB1*, *SpTAK1* and *EGFP* were synthesized using the T7 RiboMAX Express (Promega, USA) kit following the manufacturer's protocol. The concentration and quality of obtained dsRNA products were detected by NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and RNA electrophoresis. In the *in vivo* RNA interference assay, 60 μg of dsRNA for each individual was dissolved in 100 μL 1.8% (w/v) NaCl solution, and injected into juvenile crabs (weighing 50 ± 5 g) through the base of cheliped. The 1.8% NaCl solution and dsEGFP injection groups were served as the control groups. Hepatopancreas of six crabs from each group was sampled at 0 h, 48 h and 72 h post injection to measure the RNA interference efficiency by qRT-PCR.

2.9. Bacteria clearance assay in vivo

Crabs were pretreated with dsRNA-TAB1, dsRNA-TAK1, dsRNA-TAK1 + TAB1, dsRNA-EGFP and 1.8% NaCl solution for 48 h before injection with *V. alginolyticus* (100 μL, 2×10^6 CFU/mL). After 24 h of bacterial challenge, the same volume of hemolymph (100 μL) from six crabs in each group was collected, diluted with sterile anticoagulant solution at the ratio of 1:1 [35], and cultured on LB solid medium plates at 28 °C for 18 h. The number of colonies in each group was counted and recorded. The assay was performed in triplicates for each crab.

2.10. Dual-luciferase reporter assay

The ORF sequences without stop codon of *SpTAK1* and *SpTAB1* were respectively cloned into pCMV-N-MYC vectors to generate the plasmids pCMV-SpTAK1 and pCMV-SpTAB1. The dual-luciferase reporter assay was performed in HEK293T cells. 200 ng pCMV plasmid (i.e. pCMV empty vector, pCMV-SpTAK1, or pCMV-SpTAB1), 20 ng pRL-TK Renilla luciferase plasmid (Promega, USA) and 200 ng NF-κB luciferase reporter plasmid (Clontech, USA) were co-transfected into HEK293T cells in 24 wells. The pRL-TK plasmid was adopted as the internal control and the plasmid pTAL-Luc was used as the negative control. After 48 h of transfection, the cells were washed twice with PBS and then lysed for 15 min. The lysates were collected and the relatively ratios of firefly and renilla luciferase activities were detected using Dual-Luciferase® Reporter Assay System (Promega, USA) according to the manual instruction. The assay was performed in three independent transfections.

2.11. Statistical analysis

All data was presented as the mean values ± standard error of mean (SEM) of each group. Statistically significant differences were calculated by one-way ANOVA followed by Tukey test or two-way ANOVA followed by Bonferroni post test using the SPSS software (version 19.0). Differences were considered to be significant at $p < 0.05$ and extremely significant at $p < 0.01$. Graphs were generated by GraphPad Prism 5.0.

3. Results

3.1. Identification and sequence analysis

3.1.1. SpTAK1

The obtained full-length cDNA sequence of *SpTAK1* was 2, 226 bp long with a 1, 782 bp ORF that encoded a 593 amino acid (aa) protein. The sequence of *SpTAK1* was deposited in GenBank under the accession number MK319934. The theoretical pI and calculated molecular weight of *Sp*TAK1 protein were 7.06 and 65.32 kDa, respectively. As shown in Fig. 1A, the conserved domain analysis showed that *Sp*TAK1 possessed a typical S_TKc domain (Serine/Threonine protein kinases, catalytic domain) (21–269 aa). Multiple sequence alignment analysis revealed that TAK1 was conserved in diverse species especially at its N-terminal S_TKc domain (Fig. 1B). The deduced *Sp*TAK1 protein shared 84% sequence identity with TAK1 of *Penaeus vannamei* (ANR02615.1), 58% identity with TAK1 of *Trichoplusia ni* (XP_026744127.1), 39% identity with TAK1 of *Danio rerio* (NP_001018586.1), 38% identity with TAK1 of *Xenopus tropicalis* (XP_012818037.1) and *Homo sapiens* (NP_663304.1), and 34% identity with TAK1 of *Gallus gallus* (XP_015140163.1), respectively.

3.1.2. SpTAB1

The obtained full-length cDNA sequence of *SpTAB1* was 2, 433 bp long with a 1, 533 bp ORF that encoded a 510 aa protein. The sequence of *SpTAB1* was deposited in GenBank under the accession number MK319935. The theoretical pI and estimated molecular weight of *Sp*TAB1 protein were 4.95 and 56.56 kDa, respectively. Functional domain analysis indicated that the putative *Sp*TAB1 protein contained a typical PP2Cc domain (Serine/threonine phosphatases, family 2C, catalytic domain) (38–366 aa) and a potential TAK1 docking motif PYVNFDLY (481–488 aa) (Fig. 1C). Multiple sequence alignment analysis demonstrated that TAB1 was conserved in multiple species (Fig. 1D). The deduced *Sp*TAB1 protein shared 80% identity with TAB1 of *Penaeus vannamei* (AQY45916.1), 65% identity with TAB1 of *Hyalella azteca* (XP_018024349.1), 31% identity with TAB1 of *Danio rerio* (XP_002662286.4), and 34% identity with TAB1 of *Xenopus laevis* (NP_001081740.1), *Gallus gallus* (NP_001006240.2) and *Homo sapiens* (NP_006107.1).

3.2. Phylogenetic analysis

Phylogenetic analysis was employed to determine the evolutionary relationship of TAK1 and TAB1 proteins among *S. paramamosain* and other species (Fig. 2). The results demonstrated that *Sp*TAK1 and *Sp*TAB1 were clustered into their corresponding subgroups with TAK1 and TAB1 proteins from other arthropods, and had the closest relationship with *Lv*TAK1 and *Lv*TAB1 from *Litopenaeus vannamei*, which was consistent with their traditional taxonomic positions.

3.3. Expression profiles of SpTAK1 and SpTAB1 during embryonic and larval development

To study the involvement of *SpTAK1* and *SpTAB1* in crab's development process, the expression of *SpTAK1* and *SpTAB1* in the embryos/larvae was analyzed by qRT-PCR. During the embryonic development, the transcriptional levels of both *SpTAK1* and *SpTAB1* peaked at the blastula stage and gradually declined in appendage stage, eye-pigment stage, heartbeat stage and prehatching stage (Fig. 3A and B, $p < 0.05$). During the larval development, the transcripts of *SpTAK1* expressed highly at zoea I stage and moderately at zoea IV, megalopa and crab I stages, while transcriptional levels at zoea I, zoea III and zoea V stages were relatively low (Fig. 3C, $p < 0.05$). The transcripts of *SpTAB1* were predominantly expressed at zoea I stage, while the expression levels were moderate in other stages and exhibited no obvious difference (Fig. 3D, $p < 0.05$).

3.4. Tissue distribution patterns of SpTAK1 and SpTAB1 in healthy adult crabs

The constitutive expressions of *SpTAK1* and *SpTAB1* in different tissues were analyzed. The transcripts of *SpTAK1* could be detected in all examined tissues with the highest expression in the hepatopancreas and the lowest expression level in the nerve (Fig. 4A). The mRNA transcripts of *SpTAB1* were constitutively expressed in all selected tissues with an extremely high expression level in the hepatopancreas (Fig. 4B).

3.5. Expression profiles of SpTAK1 and SpTAB1 after bacterial and viral challenges

To investigate the participation of *SpTAK1* and *SpTAB1* in innate immune responses, the time-course expression profiles of *SpTAK1* and *SpTAB1* in hepatopancreas after bacterial or viral challenges were examined by qRT-PCR. Upon *V. alginolyticus* challenge, the expression of *SpTAK1* was significantly stimulated from 3 h post injection (hpi) and reached a maximum at 12 hpi (Fig. 5A). In the meanwhile, the expression of *SpTAB1* was also markedly induced at 3 hpi ($p < 0.05$), then returned to normal level at 6 hpi, followed by an increase at 12 hpi ($p < 0.05$), and finally became no significant up-regulation at 48 hpi (Fig. 5B). When challenged with Poly (I:C), the transcriptional levels of both *SpTAK1* and *SpTAB1* were dramatically up-regulated at 3 hpi ($p < 0.05$), then dropped to normal level at 6 hpi, but increased again at 12 hpi and maintained at high levels until 48 hpi (Fig. 5C and D,

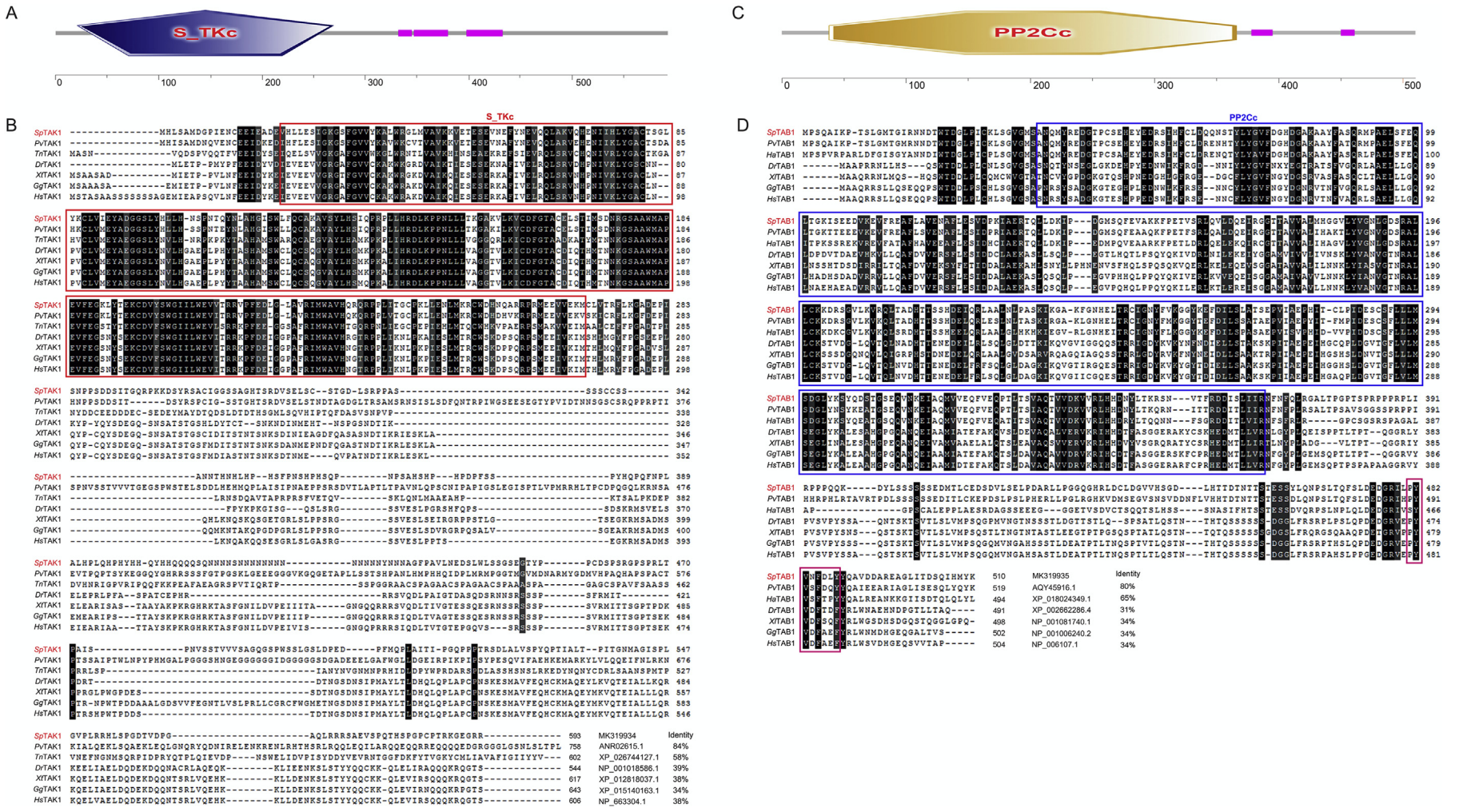

Fig. 1. Functional domain analysis and multiple sequence alignment of *Sp*TAK1 and *Sp*TAB1 proteins. (A) Schematic diagram of the domain topology of *Sp*TAK1 protein by SMART tool. The pink boxes represented low complexity regions. (B) Multiple sequence alignment of the deduced amino acid sequence of *Sp*TAK1 with TAK1 from other species, including *Penaeus vannamei* (*Pv*), *Trichoplusia ni* (*Tn*), *Danio rerio* (*Dr*), *Xenopus tropicalis* (*Xt*), *Gallus gallus* (*Gg*) and *Homo sapiens* (*Hs*). (C) Schematic diagram of the domain topology of *Sp*TAB1 protein by SMART tool. (D) Multiple sequence alignment of the deduced amino acid sequence of *Sp*TAB1 with TAB1 from other species, including *Penaeus vannamei* (*Pv*), *Tribolium castaneum* (*Tc*), *Danio rerio* (*Dr*), *Xenopus laevis* (*Xl*), *Gallus gallus* (*Gg*) and *Homo sapiens* (*Hs*). Amino acid identities and the GenBank accession numbers of all selected proteins were listed at the end of the corresponding protein sequences. The identical amino acid residues were in black shadow and the similar residues were in gray shadow. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

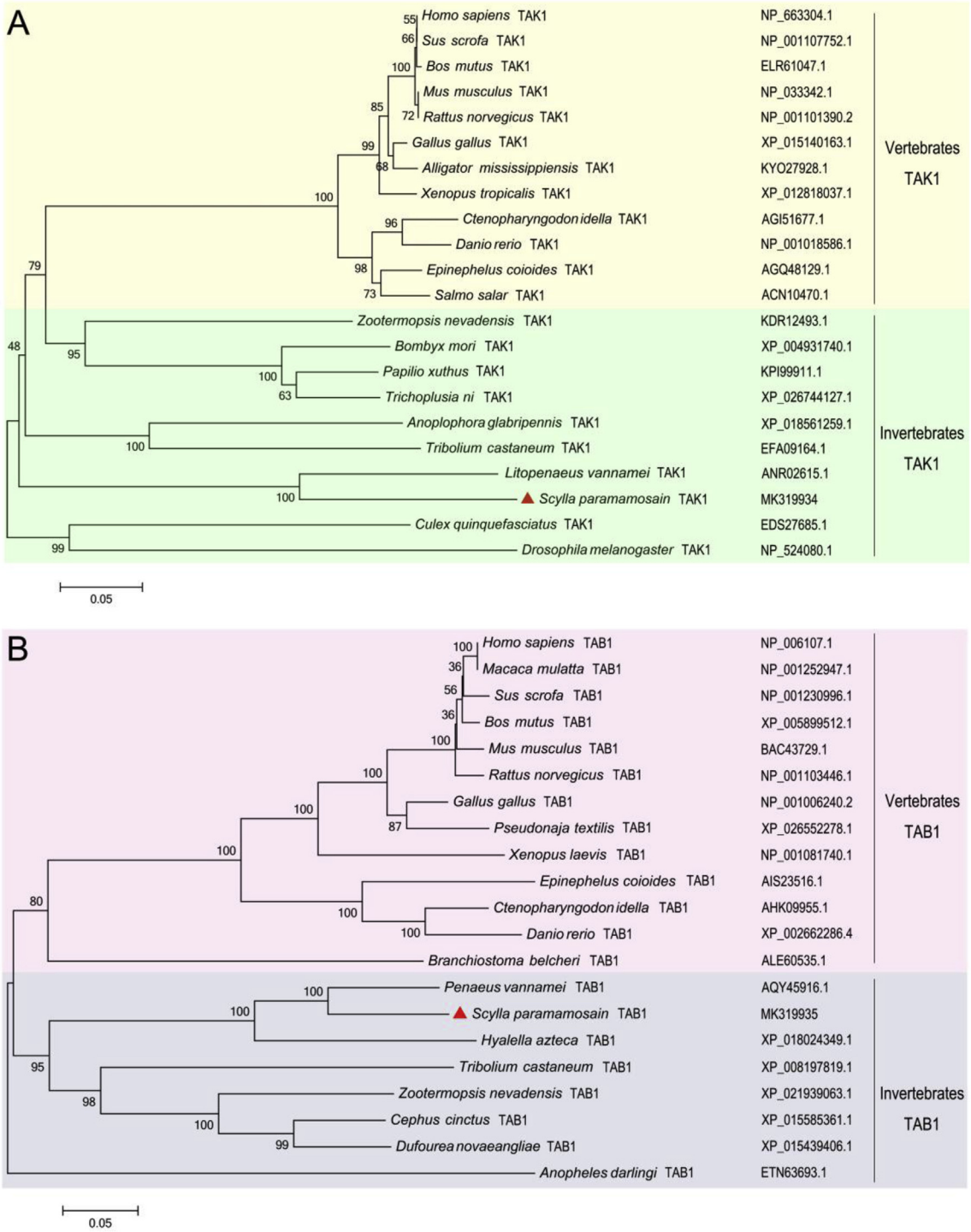

Fig. 2. The phylogenetic analysis of *Sp*TAK1 and *Sp*TAB1. (A) The phylogenetic tree of *Sp*TAK1 with TAK1 proteins from other species. (B) The phylogenetic tree of *Sp*TAB1 with TAB1 proteins from other species. The GenBank accession numbers of selected proteins were listed behind the protein names. The phylogenetic trees were generated via neighbor-joining method using MEGA 5.0 software. The value bar represented the genetic distance.

$p < 0.05$).

3.6. Silencing of SpTAK1 or SpTAB1 reduced the expressions of NF-κB pathway related genes and AMPs

RNA interference was carried out to validate whether *SpTAK1* or *SpTAB1* could regulate the expression of immune-related genes in *S. paramamosain*. As shown in Fig. 6, dsRNA-TAK1 and dsRNA-TAB1 significantly decreased the transcription of *SpTAK1* or *SpTAB1* at 48 h and 72 h after injection. Additionally, the transcriptional levels of *SpIKKβ*, *SpRelish* and six AMP genes (*SpALF1-5* and *SpCrustin*) were markedly suppressed in *SpTAK1* or *SpTAB1* silenced groups when compared with those in 1.8% NaCl or dsEGFP injection groups ($p < 0.05$).

3.7. Activation of NF-κB signaling by SpTAK1 and SpTAB1 proteins

The dual-luciferase reporter assay was further conducted to verify

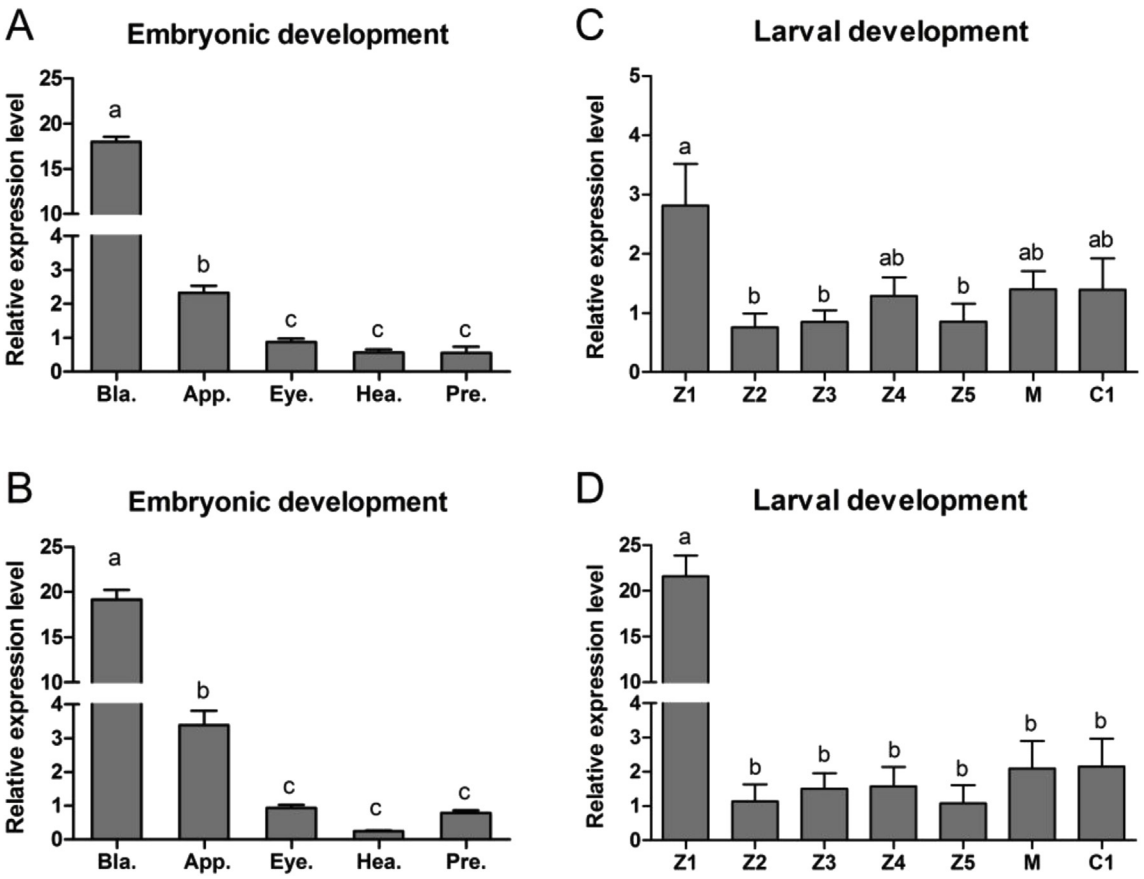

Fig. 3. The mRNA expression profiles of *SpTAK1* and *SpTAB1* during development process. (A–B) The relative transcriptional levels of *SpTAK1* (A) and *SpTAB1* (B) in different embryonic development stages. (C–D) The relative transcriptional levels of *SpTAK1* (C) and *SpTAB1* (D) in different larval development stages. The stages of embryonic development were divided into blastula stage (Bla), appendage stage (App), eye-pigment stage (Eye), heartbeat stage (Hea), and prehatching stage (Pre). The stages of larval development were divided into zoea I stage (Z1), zoea II stage (Z2), zoea III stage (Z3), zoea IV stage (Z4), zoea V stage (Z5), megalopa stage (M) and crab I stage (C1). The *β-actin* gene was used as an internal control. Each symbol and vertical bar represented the mean ± SEM (n = 5). Different letters (a, b and c) indicated significant differences ($p < 0.05$).

the regulation of *Sp*TAK1 and *Sp*TAB1 on NF-κB signaling. The results of luciferase activities displayed that both pCMV-SpTAK1 and pCMV-SpTAB1 recombinant plasmids could trigger down-stream signaling and activate the expression of mammalian NF-κB luciferase reporter vector (Fig. 7, $p < 0.001$). Notably, the NF-κB signal was significantly enhanced in cells co-transfected with pCMV-SpTAK1 and pCMV-SpTAB1 plasmids together ($p < 0.01$).

3.8. Function of SpTAK1 and SpTAB1 in the bacteria clearance capacity

The *in vivo* bacterial clearance assay was applied to validate that whether *Sp*TAK1 and *Sp*TAB1 function in the crab innate immune defense against bacterial infection. The results showed that the number of bacteria colonies in the circulating hemolymph of the dsRNA-TAK1 or dsRNA-TAB1 groups were remarkably higher than those in dsRNA-EGFP group and 1.8% NaCl solution group (Fig. 8, $p < 0.05$).

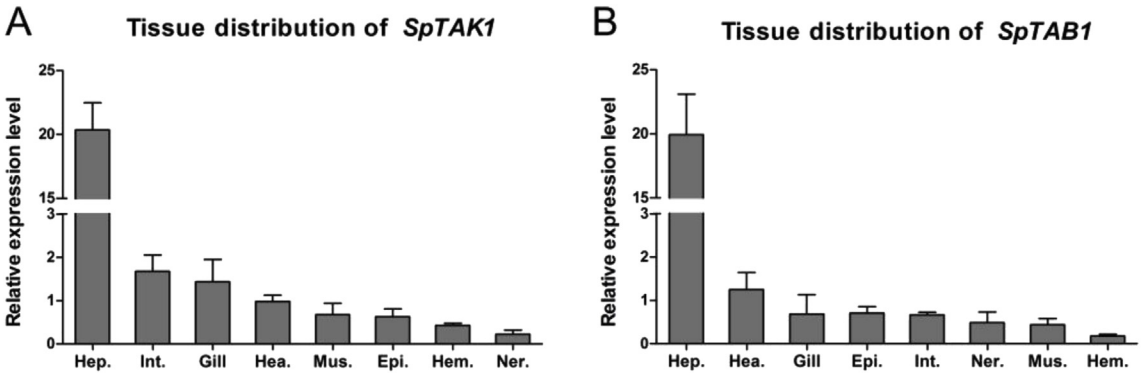

Fig. 4. Tissue distribution of *TAK1* (A) and *TAB1* (B) in adult *S. paramamosain*. Different tissues including the hepatopancreas (Hep.), intestine (Int.), gill (Gil.), heart (Hea.), muscle (Mus.), epicdermis (Epi.), hemocytes (Hem.) and nerve (Ner.) were collected from healthy *S. paramamosain* to extract total RNA for tissue distribution analysis. The *β-actin* gene was used as an internal control. Each symbol and vertical bar represented the mean ± SEM (n = 4).

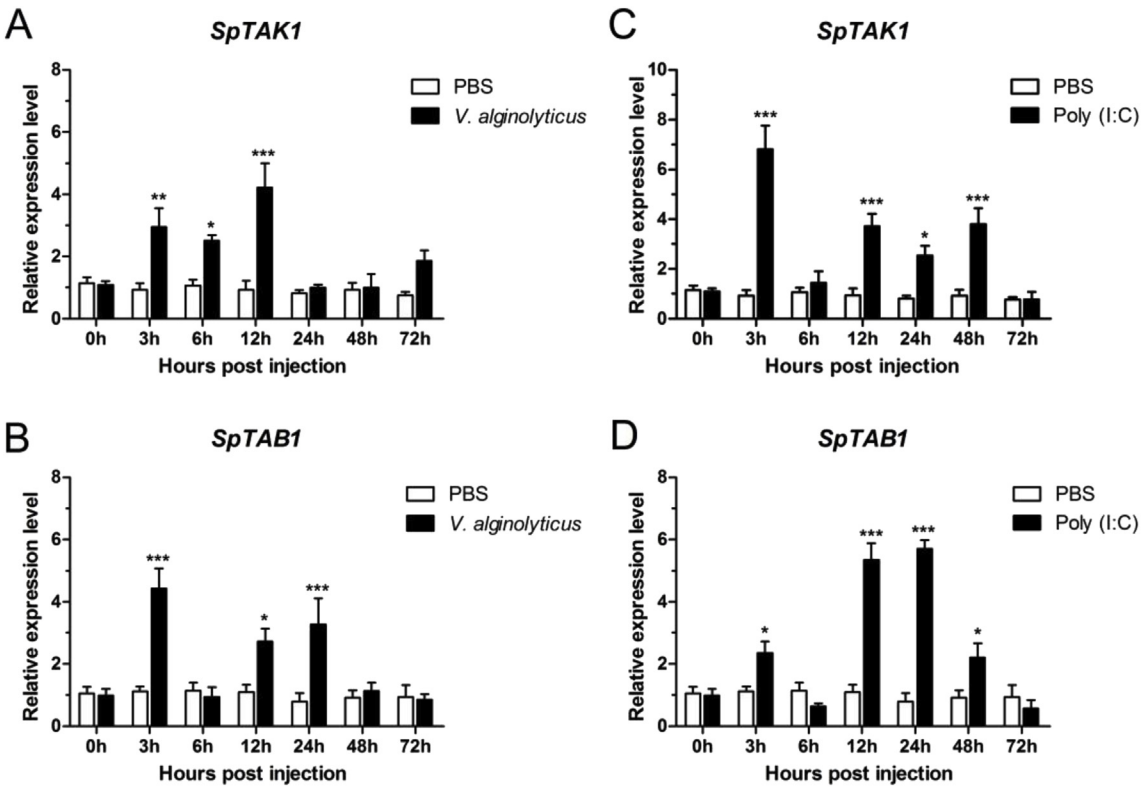

Fig. 5. The mRNA expression profiles of *SpTAK1* and *SpTAB1* upon immune challenge. (A–B) Temporal expression of *SpTAK1* and *SpTAB1* in hepatopancreas at different time points after challenge with bacteria *V. alginolyticus*. (C–D) Temporal expression of *SpTAK1* and *SpTAB1* in hepatopancreas at different time points after stimulation with virus analogue Poly (I:C). The *β-actin* gene was used as an internal control and the PBS group was served as the control group. Data was shown as mean ± SEM (n = 6). The asterisks indicated significant differences between challenged groups and control groups (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

4. Discussion

TAK1, a member of the MAP3K family, is a key signaling molecule in multiple biological activities, including innate immunity, development and cell survival [9,10,36]. TABs act as activators and adaptors that specifically activate TAK1 and trigger the downstream signaling events [16–18]. TAK1, TAB1 and TAB2 from *L. vannamei* have been reported to modulated the expressions of AMPs in shrimp and *Drosophila* S2 cells [29–31]. However, the detailed regulatory mechanisms of TAK1 and TABs in innate immunity are still elusive in crustaceans.

In the present study, the homologs of TAK1 and TAB1 were identified and characterized from mud crab *S. paramamosain*. The deduced *Sp*TAK1 possessed a conserved S_TKc domain at the N-terminus (Fig. 1), which is critical for TAK1 to activate IKKs and MAPKs [19]. In addition, a typical PP2Cc domain was identified in putative *Sp*TAB1 protein (Fig. 1). Previous studies demonstrated that a C-terminal motif of PYVDXA/TXF was conserved in TAB1 proteins from different species, which functioned as the specific docking site for TAK1 [37]. The PYVNFDLY motif was also found at the C-terminus of *Sp*TAB1 and shared high homology with other TAB1 proteins, suggesting a potential interaction between *Sp*TAB1 and *Sp*TAK1. Moreover, the phylogenetic analysis revealed that *Sp*TAK1 and *Sp*TAB1 grouped together with TAK1 and TAB1 proteins from other arthropods, respectively (Fig. 2). These results demonstrated that *Sp*TAK1 and *Sp*TAB1 belonged to the conservative TAK1 and TAB1 family individually.

TAB1 was firstly identified as an activator of the TAK1 in TGF-β signal transduction [38]. Previous reports have shown that TAK1 mutant mouse embryos exhibit defects in the developing vasculature of the yolk sac and embryo proper [39], and ectopic expression of xTAK1 in early *Xenopus* embryos could induce cell death [40]. However, the biological roles of TAK1 and TAB1 are poorly understood in crustaceans. In this study, the expression profiles of *SpTAK1* and *SpTAB1* in development process were investigated. Results showed both *SpTAK1* and *SpTAB1* were predominantly expressed at blastula stage and zoea I stage (Fig. 3), suggesting that *SpTAK1* and *SpTAB1* might play essential roles in early embryonic development and early larval development. Interestingly, the expression patterns of *SpTAK1* and *SpTAB1* were similar to that of TGF-β type I receptor in *S. paramamosain* embryos and larvae [41], implying a possible interaction of TAK1/TAB1 with TGF-β signaling in crabs.

Tissue expression analysis revealed that *SpTAK1* and *SpTAB1* transcripts were constitutively expressed in all examined tissues of mud crabs, with the predominant expression in the hepatopancreas (Fig. 4), which is an important tissue in crustaceans for metabolism and immunity. Since TAK1 is known as a crucial signal molecule in immunological contexts [36], the temporal expression profiles of *SpTAK1* and *SpTAB1* upon *V. alginolyticus* and Poly (I:C) stimulation were examined, results revealed that the transcripts of both *SpTAK1* and *SpTAB1* were dramatically induced after the bacterial and viral challenges (Fig. 5). Similar expression pattern could be found in Pacific shrimps (*L. vannamei*) challenged with LPS, *V. parahaemolyticus* or Poly (I:C) [29,31]. These results suggested TAK1/TAB1 might play ancestral roles in innate immune responses against bacterial and viral infection in crustaceans.

TAK1 regulates inflammation and innate immunity through activating specific downstream signaling events [10,42]. In mammals, the TAK1/TAB1 complex stimulates the phosphorylation and degradation of IκB, which ultimately results in the activation of NF-κB [15,19,20]. To illuminate the regulatory roles of *SpTAK1* and *SpTAB1* in innate immune signaling cascades, the expression levels of important immune genes including IKKs (*SpIKKβ* and *SpIKKε*) and NF-κBs (*SpRelish* and *SpDorsal*) were examined in *SpTAK1*- or *SpTAB1*-knockdowned crabs. When *SpTAK1* or *SpTAB1* was silenced, the transcriptional levels of *SpIKKβ* and *SpRelish* significantly decreased, while no significant

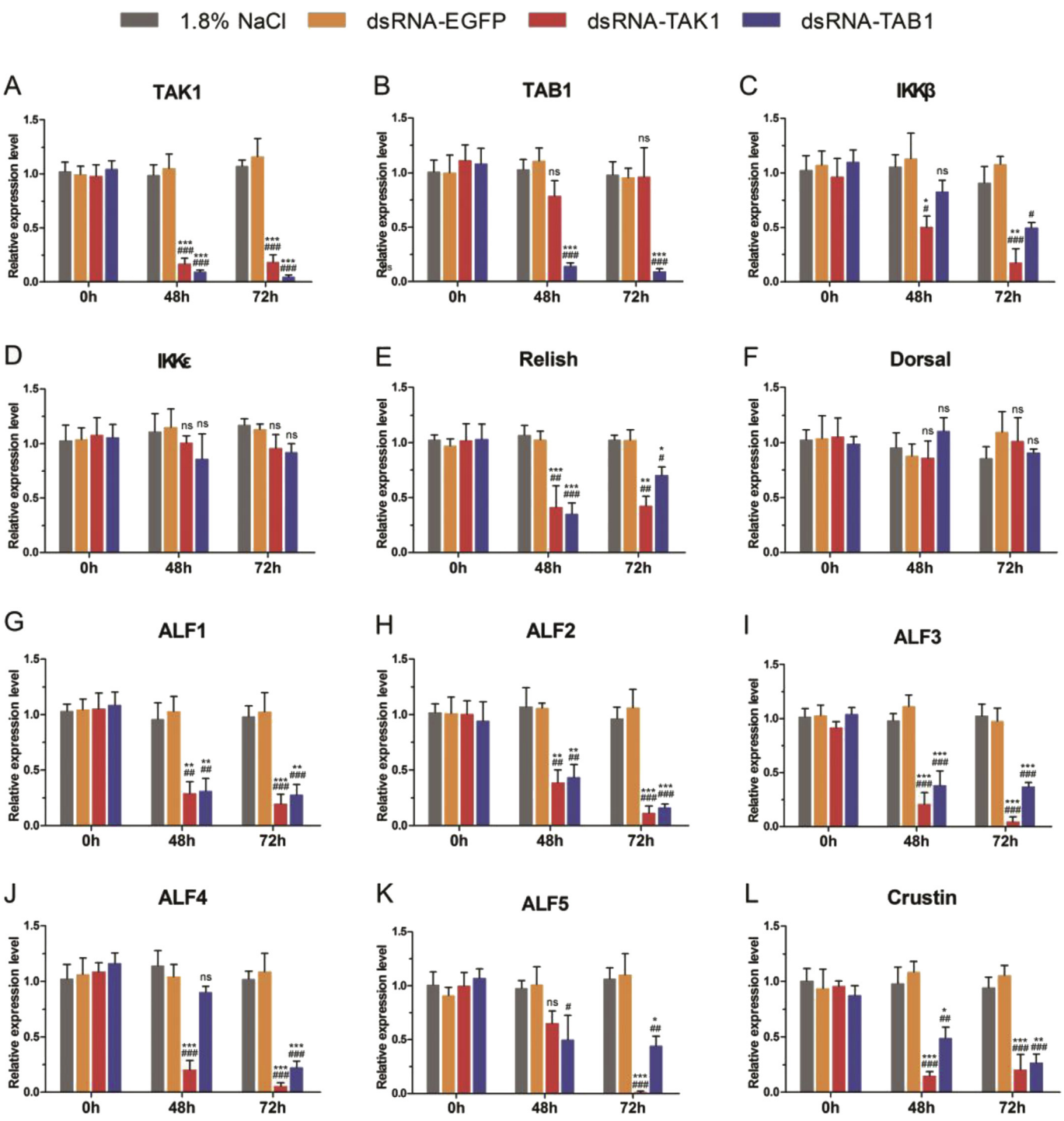

Fig. 6. Expression analysis of immune genes and AMPs in *SpTAK1*-silenced or *SpTAB1*-silenced crabs. Each symbol and vertical bar represents the mean ± SEM (n = 6). The *β-actin* gene was used as an internal control. The asterisks indicated significant differences between challenged groups and 1.8% NaCl group (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). The hash symbols indicated significant differences between challenged groups and dsRNA-EGFP group (#$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$).

difference in the expression levels of *SpIKKε* and *SpDorsal* was found among the *SpTAK1* or *SpTAB1* silenced groups and control groups (Fig. 6). Notably, *SpIKKβ* and *SpRelish* are two important components of crab IMD signaling pathway [43,44], these results indicated that TAK1/TAB1 complex was associated with the IMD signaling in crabs.

The IMD pathway regulates the activation of NF-κB and controls the production of most of AMPs in invertebrates [7]. Previous studies had shown that overexpression of *Lv*TAK1 or *Lv*TAB1 in *Drosophila* S2 cells could strongly induce the promoter activity of *Diptericin*, a typical AMP which was used to read out of the activation of IMD pathway [29,31]. In this study, the expressions of six AMPs including *SpALF1-5* and *SpCrustin* were consistently suppressed when *SpTAK1* or *SpTAB1* were knocked down (Fig. 6). In bony fish (*Larimichthys crocea*), the interaction of TAK1 and TAB1 enhanced cytokine release via modulating NF-κB activation [45]. The dual-luciferase reporter assay further verified the regulatory roles of *Sp*TAK1 and *Sp*TAB1 on NF-κB signaling (Fig. 7). Notably, the luciferase activity was significantly enhanced when *Sp*TAK1 and *Sp*TAB1 were co-transfected, implying that the function of

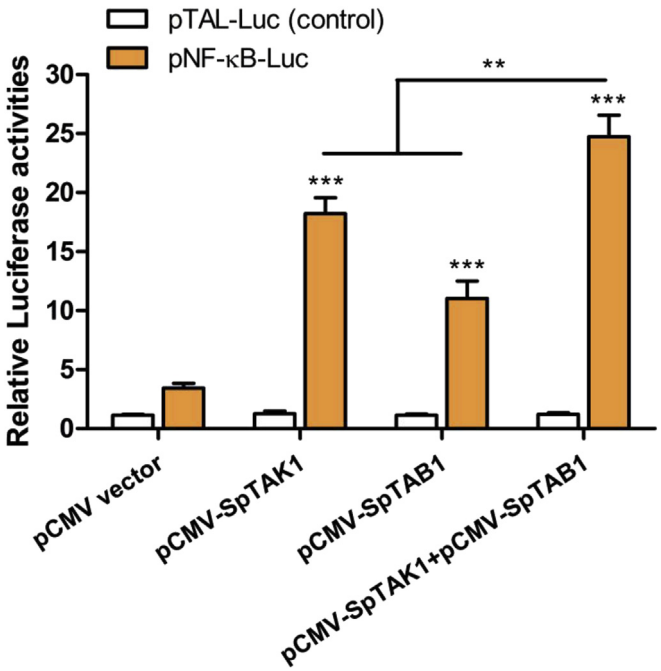

Fig. 7. The activation of NF-κB luciferase reporter by *Sp*TAB1 and *Sp*TAK1 in HEK293T cells. The cells were co-transfected with the same amount of pCMV empty vector, pCMV-SpTAK1 or pCMV-SpTAB1 together with the NF-κB luciferase reporter vector and the internal control *Renilla* expression vector for 48 h. The pCMV empty vector and the pTAL luciferase reporter vector were used as negative controls. Data was shown as mean ± SEM (n = 3, $^{**}p < 0.01$ and $^{***}p < 0.001$).

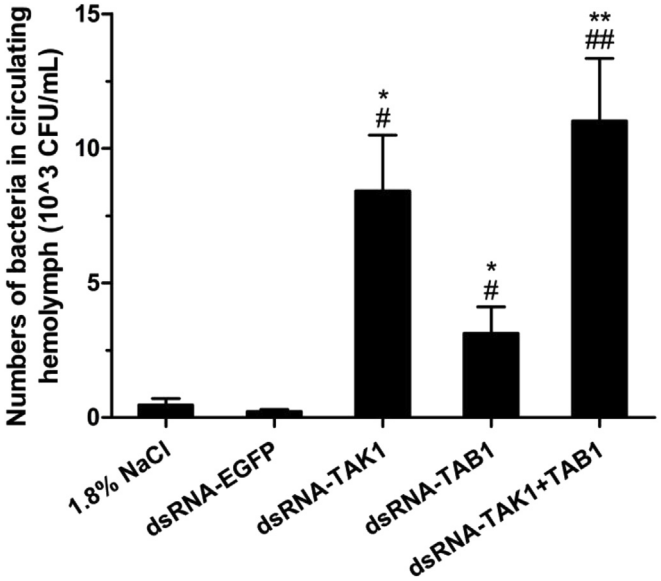

Fig. 8. *In vivo* bacteria clearance assay. Same volume (100 μL) of hemolymph was extracted from mud crabs in different treatment groups, diluted with sterile anticoagulant solution at the ratio of 1:1, and cultured on LB solid medium plates at 28 °C for 18 h. The number of colonies was counted and recorded. Data was shown as mean values ± SEM (n = 6). The asterisks indicated significant differences between challenged groups and 1.8% NaCl group ($^{*}p < 0.05$ and $^{**}p < 0.01$). The hash symbols indicated significant differences between challenged groups and dsRNA-EGFP group ($\#p < 0.05$ and $\#\#p < 0.01$). The assay was performed in triplicates for each crab.

*Sp*TAK1/*Sp*TAB1 complex was evolutionally well conserved. The secretion of AMPs into hemolymph is a characteristic of humoral response against intruders in invertebrates [1,7,21]. The *in vivo* bacteria clearance assay was applied to validate whether the down-regulation of AMPs mediated by silencing *SpTAK1* or *SpTAB1* would affect the innate immunity of crabs. Results exhibited that the bacteria clearance capacity was markedly impaired in *SpTAK1* or *SpTAB1* silenced crabs (Fig. 8), indicating the protective role of *Sp*TAK1 and *Sp*TAB1 in crab innate immune responses against bacteria invasion. Taken together, these results suggested that *Sp*TAK1 together with *Sp*TAB1 might regulate the IMD−NF−κB signaling pathway and the expressions of AMPs to influence the innate immune responses of crabs. Actually, the activated TAK1/TAB1 complex could result in the activation of various downstream factors [46]. The ability of TAB1 interacting with p38 has been identified in *L. vannamei* [29], which means TAB1 could have a role in regulating the MAPK signaling in shrimps to protect host from pathogen invasion. However, whether TAB1 could directly activate p38 in crabs is still obscure and the interaction of TAK1/TABs with other signaling molecules needs further investigation.

In conclusion, the homologs of TAK1 and TAB1 were firstly identified and characterized from mud crab *S. paramamosain*. The distinct expression patterns of *SpTAK1* and *SpTAB1* during embryonic and larval development processes suggested their conserved functions in development in crustaceans, and the significant induction of *SpTAK1* and *SpTAB1* after pathogen challenges indicated their possible participation in innate immune responses. The bacterial clearance assay further verified that *SpTAK1* and *SpTAB1* played essential roles in crabs' innate immune defense system. Moreover, the *in vivo* RNA interference assay and dual luciferase reporter experiments demonstrated that *SpTAK1* and *SpTAB1* might regulate the expressions of AMPs through activating the IMD−NF−κB signaling pathway. These findings provided new insights into the TAK1/TAB1-mediated immune signaling cascades in crustaceans, which might pave a way to develop more effective strategies for prevention and control of crab diseases.

Acknowledgements

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References

[1] T.J. Little, D. Hultmark, A.F. Read, Invertebrate immunity and the limits of mechanistic immunology, Nat. Immunol. 6 (2005) 651–654.
[2] R. Medzhitov, C.A. Janeway, Innate immunity: the virtues of a nonclonal system of recognition, Cell 91 (1997) 295–298.
[3] S. Akira, S. Uematsu, O. Takeuchi, Pathogen recognition and innate immunity, Cell 124 (2006) 783–801.
[4] G. Ennio De, T.S. Paul, T. Phoebe, M.R. Gerald, L. Bruno, The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*, EMBO J. 21 (2002) 2568–2579.
[5] S. Minakhina, R. Steward, Nuclear factor-kappa B pathways in *Drosophila*, Oncogene 25 (2006) 6749–6757.
[6] S. Valanne, J.-H. Wang, M. Ramet, The *Drosophila* Toll signaling pathway, J. Immunol. 186 (2011) 649–656.
[7] H. Myllymaki, S. Valanne, M. Ramet, The *Drosophila* imd signaling pathway, J. Immunol. 192 (2014) 3455–3462.
[8] K. Yamaguchi, K. Shirakabe, H. Shibuya, K. Irie, I. Oishi, N. Ueno, T. Taniguchi, E. Nishida, K. Matsumoto, Identification of a member of the MAPKKK family as a potential mediator of TGF-β signal transduction, Science 270 (1995) 2008–2011 (80-.).
[9] S.R. Mihaly, J. Ninomiya-Tsuji, S. Morioka, TAK1 control of cell death, Cell Death Differ. 21 (2014) 1667–1676.
[10] S. Sato, H. Sanjo, K. Takeda, J. Ninomiya-Tsuji, M. Yamamoto, T. Kawai, K. Matsumoto, O. Takeuchi, S. Akira, Essential function for the kinase TAK1 in innate and adaptive immune responses, Nat. Immunol. 6 (2005) 1087–1095.
[11] V. Baud, M. Karin, Signal transduction by tumor necrosis factor and its relatives, Trends Cell Biol. 11 (2001) 372–377.
[12] S. Akira, K. Takeda, Toll-like receptor signalling, Nat. Rev. Immunol. 4 (2004) 499–511.
[13] Z. Jiang, J. Ninomiya-Tsuji, Y. Qian, K. Matsumoto, X. Li, Interleukin-1 (IL-1) receptor-associated kinase-dependent IL-1-induced signaling complexes phosphorylate TAK1 and TAB2 at the plasma membrane and activate TAK1 in the cytosol,

Mol. Cell Biol. 22 (2002) 7158–7167.
[14] K. Shirakabe, K. Yamaguchi, H. Shibuya, K. Irie, S. Matsuda, T. Moriguchi, Y. Gotoh, K. Matsumoto, E. Nishida, TAK1 mediates the ceramide signaling to stress-activated protein kinase/c-jun N-terminal, J. Biol. Chem. 272 (1997) 8141–8144.
[15] H. Sakurai, H. Miyoshi, W. Toriumi, T. Sugita, Functional interactions of transforming growth factor β-activated kinase 1 with IκB kinases to stimulate NF-κB activation, J. Biol. Chem. 274 (1999) 10641–10648.
[16] H. Shibuya, K. Yamaguchi, K. Shirakabe, A. Tonegawa, Y. Gotoh, N. Ueno, K. Irie, E. Nishida, K. Matsumoto, TAB1 : an activator of the TAK1 MAPKKK in TGF-β signal transduction, Science 272 (1996) 1179–1182 (80-.).
[17] P.C.F. CHEUNG, A.R. NEBREDA, P. COHEN, TAB3, a new binding partner of the protein kinase TAK1, Biochem. J. 378 (2004) 27–34.
[18] G. Takaesu, S. Kishida, A. Hiyama, K. Yamaguchi, H. Shibuya, K. Irie, J. Ninomiya-Tsuji, K. Matsumoto, TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway, Mol. Cell. 5 (2000) 649–658.
[19] J. Ninomiya-Tsuji, K. Kishimoto, A. Hiyama, J.I. Inoue, Z. Cao, K. Matsumoto, The kinase TAK1 can activate the NIK-IκB as well as the MAP kinase cascade in the IL-1 signalling pathway, Nature 398 (1999) 252–256.
[20] W. Chen, D. Li, H. Mei, R.A. Giridhar, I. Jun-ichiro, J.C. Zhijian, TAK1 is a ubiquitin-dependent kinase of MKK and IKK, Nature 412 (2001) 1–11.
[21] B. Lemaitre, J. Hoffmann, The host defense of *Drosophila melanogaster*, Annu. Rev. Immunol. 25 (2007) 697–743.
[22] M. Gottar, V. Gobert, T. Michel, M. Belvin, G. Duyk, J.A. Hoffmann, D. Ferrandon, J. Royet, The *Drosophila* immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein, Nature 416 (2002) 640–644.
[23] P. Georgel, S. Naitza, C. Kappler, D. Ferrandon, D. Zachary, C. Swimmer, C. Kopczynski, G. Duyk, J.M. Reichhart, J.A. Hoffmann, *Drosophila* immune deficiency (IMD) is a death domain protein that activates antibacterial defense and can promote apoptosis, Dev. Cell 1 (2001) 503–514.
[24] S. Hu, X. YangdFADD, A novel death domain-containing adapter protein for the *Drosophila* caspase DREDD, J. Biol. Chem. 275 (2000) 30761–30764.
[25] S. Vidai, R.S. Khush, F. Leulier, P. Tzou, M. Nakamura, B. Lemaitre, Mutations in the *Drosophila* dTAK1 gene reveal a conserved function for MAPKKKs in the control of rel/NF-κB-dependent innate immune responses, Genes Dev. 15 (2001) 1900–1912.
[26] A. Kleino, S. Valanne, J. Ulvila, J. Kallio, H. Myllymäki, H. Enwald, S. Stöven, M. Poidevin, R. Ueda, D. Hultmark, B. Lemaitre, M. Rämet, Inhibitor of apoptosis 2 and TAK1-binding protein are components of the *Drosophila* Imd pathway, EMBO J. 24 (2005) 3423–3434.
[27] D. Ertürk-Hasdemir, M. Broemer, F. Leulier, W.S. Lane, N. Paquette, D. Hwang, C.-H. Kim, S. Stöven, P. Meier, N. Silverman, Two roles for the *Drosophila* IKK complex in the activation of Relish and the induction of antimicrobial peptide genes, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 9779–9784.
[28] M.S. Dushay, B. Asling, D. Hultmark, Origins of immunity: Relish, a compound Rel-like gene in the antibacterial defense of *Drosophila*, Proc. Natl. Acad. Sci. Unit. States Am. 93 (1996) 10343–10347.
[29] S. Wang, M. Li, B. Yin, H. Li, B. Xiao, K. Lǔ, Z. Huang, S. Li, J. He, C. Li, Shrimp TAB1 interacts with TAK1 and p38 and activates the host innate immune response to bacterial infection, Mol. Immunol. 88 (2017) 10–19.
[30] S. Wang, H. Li, Z. Qian, X. Song, Z. Zhang, H. Zuo, X. Xu, S. Weng, J. He, C. Li, Identification and functional characterization of the TAB2 gene from *Litopenaeus vannamei*, Fish Shellfish Immunol. 46 (2015) 206–216.
[31] S. Wang, H. Li, K. Lü, Z. Qian, S. Weng, J. He, C. Li, Identification and characterization of transforming growth factor β-activated kinase 1 from *Litopenaeus vannamei* involved in anti-bacterial host defense, Fish Shellfish Immunol. 52 (2016) 278–288.
[32] L.B. Ma, F.Y. Zhang, C.Y. Ma, Z.G. Qiao, *Scylla paramamosain* (Estampador) the most common mud crab (Genus *Scylla*) in China: evidence from mtDNA, Aquacult. Res. 37 (2006) 1694–1698.
[33] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (2001) 402–408.
[34] J.W. Davis, R.K. Sizemore, Incidence of *Vibrio* species associated with blue crabs (*Callinectes sapidus*) collected from Galveston Bay, Texas, Appl. Environ. Microbiol. 43 (1982) 1092–1097.
[35] Y.L. Zhou, W. Bin Gu, D.D. Tu, Q.H. Zhu, Z.K. Zhou, Y.Y. Chen, M.A. Shu, Hemocytes of the mud crab *Scylla paramamosain*: cytometric, morphological characterization and involvement in immune responses, Fish Shellfish Immunol. 72 (2018) 459–469.
[36] H.-H. Liu, M. Xie, M.D. Schneider, Z.J. Chen, Essential role of TAK1 in thymocyte development and activation, Proc. Natl. Acad. Sci. Unit. States Am. 103 (2006) 11677–11682.
[37] K. Ono, T. Ohtomo, S. Sato, Y. Sugamata, M. Suzuki, N. Hisamoto, J. Ninomiya-Tsuji, M. Tsuchiya, K. Matsumoto, An evolutionarily conserved motif in the TAB1 C-terminal region is necessary for interaction with and activation of TAK1 MAPKKK, J. Biol. Chem. 276 (2001) 24396–24400.
[38] J.L. Jadrich, M.B. O'Connor, E. Coucouvanis, Expression of TAK1, a mediator of TGF-β and BMP signaling, during mouse embryonic development, Gene Expr. Patterns 3 (2003) 131–134.
[39] J.L. Jadrich, M.B. O'Connor, E. Coucouvanis, The TGF-β activated kinase TAK1 regulates vascular development *in vivo*, Development 133 (2006) 1529–1541.
[40] H. Shibuya, H. Iwata, N. Masuyama, Y. Gotoh, K. Yamaguchi, K. Irie, K. Matsumoto, E. Nishida, N. Ueno, Role of TAK1 and TAB1 in BMP signaling in early *Xenopus* development, EMBO J. 17 (1998) 1019–1028.
[41] Y.L. Zhou, C. Wang, W. Bin Gu, Q. hui Zhu, L.Z. Wang, Z.K. Zhou, Z.P. Liu, Y.Y. Chen, M.A. Shu, Identification and functional analysis of transforming growth factor-β type I receptor (TβR1) from *Scylla paramamosain*: the first evidence of TβR1 involved in development and innate immunity in crustaceans, Dev. Comp. Immunol. 88 (2018) 144–151.
[42] J.R. Delaney, M. Mlodzik, TGF-β activated kinase-1: new insights into the diverse roles of TAK1 in development and immunity, Cell Cycle 5 (2006) 2852–2855.
[43] M. Jiang, D.D. Tu, W. Bin Gu, Y.L. Zhou, Q.H. Zhu, X.L. Guo, M.A. Shu, Identification and functional analysis of inhibitor of NF-κB kinase (IKK) from *Scylla paramamosain*: the first evidence of three IKKs in crab species and their expression profiles under biotic and abiotic stresses, Dev. Comp. Immunol. 84 (2018) 199–212.
[44] Y.L. Zhou, L.Z. Wang, W. Bin Gu, C. Wang, Q.H. Zhu, Z.P. Liu, Y.Y. Chen, M.A. Shu, Identification and functional analysis of immune deficiency (IMD) from *Scylla paramamosain*: the first evidence of IMD signaling pathway involved in immune defense against bacterial infection in crab species, Fish Shellfish Immunol. 81 (2018) 150–160.
[45] S.Y. Bao, Q.X. Sun, C.L. Yao, The interaction of TAK1 and TAB1 enhances LPS-induced cytokine release via modulating NF-κB activation (Larimichthys crocea), Fish Shellfish Immunol. 74 (2018) 450–458.
[46] L. Dai, C. Aye Thu, X.Y. Liu, J. Xi, P.C.F. Cheung, TAK1, more than just innate immunity, IUBMB Life 64 (2012) 825–834.